Development of Anti-allergic Lead(s) from Ayurvedic Plants through the Suppression of PKCδ Dependent Signaling Pathway

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March, 2016

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Acknowledgement

Combine efforts is required to accomplish a venture to be successful. This is my immense pleasure to express my gratitude to all the individuals who contributed a lot to complete my research project.

I take the opportunity to articulate my heartiest gratitude to Dr. Hiroyuki Fukui, Emeritus Professor, Department of Molecular Studies for Incurable Diseases, Institute of Biomedical Sciences, Tokushima University, for his affectionate encouragement to pursue this research works. This is an honor and unique opportunity for me to work under his supervision.

I convey my sincere gratefulness to Dr. Hiroyuki Mizuguchi, Associate Professor, Department of Molecular Pharmacology, Institute of Biomedical Sciences, Tokushima University, for his continuous cooperation and valuable suggestions to make the project successful. His constructive criticisms build up my devotion, deep attitude in learning, and strong sense of responsibility towards research. I deliver my deepest honor to Dr. Hiroyuki Mizuguchi for his sincere supervision throughout the project.

I express my earnest gratitude to Dr. Hisao Nemoto, Associate Professor, Department of Pharmaceutical Chemistry, Institute of Biomedical Sciences, Tokushima University, for his demonstrative guidance towards synthesis of compounds.

I am thankful to Professor Yoshiki Kashiwada, Department of Pharmacognosy, Institute of Biomedical Sciences, Tokushima University, for his cordial help regarding structure determination of my novel compound.

I am grateful to Dr. Asish Kumar Das, Professor, Pharmacy Discipline, Khulna University, Bangladesh and Dr. Sanmoy Karmakar, Professor, Department of

Pharmaceutical Technology, Jadavpur University, India for their beloved support and friendly cooperation.

I express my warm and loving gratitude towards my family members who continuously support and encourage me to make my project to a successful end.

My heartiest thanks to my lab members who helped me a lot to perform my work successfully.

I acknowledge my gratefulness to MEXT for their financial support from Monubukagaksho Scholarship.

Last but not the least I extend my heartfelt gratitude to all the office staffs, Faculty of Pharmaceutical Sciences for their cordial help during my stay in the Tokushima University.

Manik Chandra Shill

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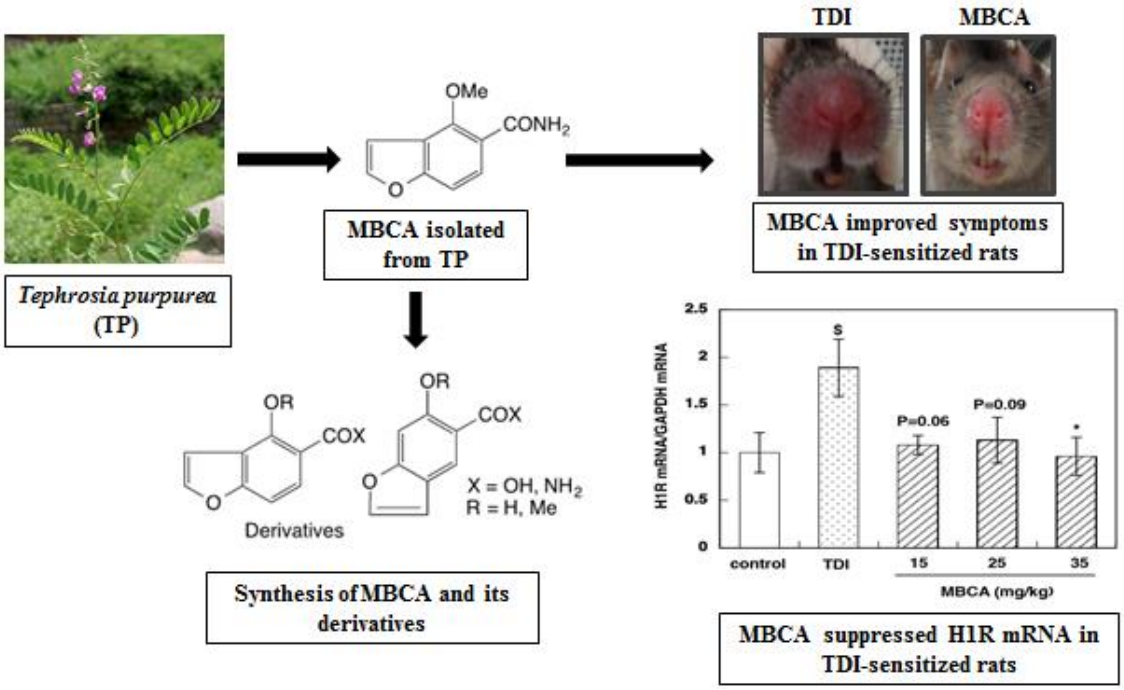


Abstract

Allergic rhinitis (AR) is an inflammatory disease caused by the activation of mucosal mast cells located in the epithelium of the nasal cavity, and is characterized by sneezing, itching, congestion, rhinorrhea, and loss of the sense of smell. Histamine is one of the key biological players in allergic diseases including AR. Up-regulation of histamine H_1 receptor (H1R) was observed in AR patients. A positive correlation between the expression of H1R mRNA and severity of allergic symptoms has also been reported in the patients with pollinosis and toluene-2,4-diisocyanate (TDI)-sensitized allergic model rats. Accordingly, compound(s) that suppress H1R gene expression might be a good therapeutics in AR. Although advances in biological and chemical technology have led to several modern medicines for palliative treatment of AR, no conclusive therapeutic agent(s) for treating AR has yet been identified. As a part of our ongoing search for anti-allergic compound(s), we have studied six Ayurvedic plants and selected *Tephrosia purpurea* (TP) for the isolation of lead compound(s) through the suppression of H1R gene expression in a PKCδ dependent signaling pathway.

A novel naturally occurring compound 4-methoxybenzofuran-5-carboxamide (MBCA) was isolated from the plant TP. The chemical synthesis of this compound confirmed its structure, and preliminary biological results showed its suppressive activity towards H1R gene expression. One isomer and four derivatives were also synthesized and in-vitro activity showed that methylation and amide formation is very important for the suppression of H1R gene expression. Pretreatment with various concentrations of MBCA dose dependently suppressed PMA- and histamine-induced H1R mRNA expression in HeLa cells. Additionally, suppression of histamine-induced

H1R mRNA expression by MBCA (50 μM) was comparable with that of PKCδ-selective inhibitor rottlerin and the conventional antihistamines chlorpheniramine and epinastine. MBCA also dose-dependently suppressed PMA-induced up-regulation of H1R protein expression. Moreover, MBCA suppressed PMA-induced phosphorylation of PKCδ at Tyr^{311} and inhibit PMA-induced translocation of PKCδ from cytosol to the Golgi. The compound improved allergic symptoms in TDI-sensitized rats and significantly suppressed the elevation of H1R mRNA. The compound also exhibited significant suppression of Th2 cytokine mRNAs (IL-4, IL-5, IL-9 and IL-13) in TDI-sensitized rats. Hence, MBCA improved allergic symptoms in TDI-sensitized rats through the disruption of histamine-cytokine network. The inhibition of PKCδ activation played the underlying molecular mechanism for H1R gene expression.

Section -1

Introduction

1.1 Allergy and its socio-economic impact:

Allergic rhinitis (AR) is an inflammatory disease caused by IgE-mediated activation of mucosal mast cells located in the epithelium of the nasal cavity, and is characterized by sneezing, itching, congestion, rhinorrhea, and loss of the sense of smell [1]. Several alternative names of the disease frequently used in publications include allergic rhinitis, nasal allergy, nasal hypersensitivity, and pollinosis. Allergic rhinitis is a global health problem that causes major illness and disability worldwide. It affects not only social life but also personal life including sleep, school, and work [2-7]. In 1996, the overall direct costs of treating allergic rhinitis exceeded $3 billion with an additional $4 billion for treating comorbidities that are triggered by rhinitis. To this cost other indirect costs such as lowered productivity and lost work time should be added. In the United States alone, the number of lost workdays is estimated as ~ 3.5 million a year [8-9].

1.2 Role of histamine and cytokines and their receptors in allergy:

Histamine is an important mediator in the initiation and the development of allergic reactions. It is the amine produced by the decarboxylation of histidine, stored in the secretory granules of all mature mast cells including connective tissues, mucosal membranes, skin, and basophils; and its action is mediated through four distinct receptors including H1, H2, H3, and H4 receptors [10, 11]. Studies have revealed that activation of H1R by histamine is responsible for the symptoms of allergic rhinitis, including sneezing, watery rhinorrhea, and nasal itching, and antihistamines are capable of controlling nasal hypersensitivity symptoms in nasal allergy [12 – 15].

Allergic reaction is also characterized by the disruption of helper T cell type 1 / 2 (Th1/Th2) balance toward a pronounced Th2 profile. Th1/Th2 imbalance in the immune system towards the Th2 responses results in the clinical expression of nasal allergy and asthma [16]. Th2-cytokines, especially IL-4, IL-5, and IL-13, may play a vital role in the development and maintenance of allergic responses [17 – 21].

Increasing experimental evidences suggest the existence and important role of the histamine–cytokine network in allergic inflammation, in which histamine influences the expression and actions of several cytokines and some cytokines modulate the production and release of histamine [22 – 24].

1.3 Limitations of current therapeutics for allergy:

Numbers of antihistamines are available for the treatment of various allergic disorders. Most of these antihistamines are synthesized as selective and competitive antagonists of the histamine H1 receptor (H1R) [25, 26]. But many of the classical H1-antihistamines at therapeutic dosage showed side effects in central nervous system (CNS) [27 – 29] and cardiovascular disorder [30].

Thus H1 antihistamines, leukotriene antagonists and steroids all alleviate allergic rhinitis but no conclusive treatment is totally effective and that the different treatments target the different symptoms and signs of the disease. It is not surprising therefore that the ideal drug treatment for this disease has yet to be discovered.

1.4 Purpose of the Study:

WHO estimates, traditional, complementary, alternative, or non-conventional medicines are used by 70–95% of global population particularly in developing countries for their healthcare. Several traditional healthcare systems exist in India from centuries and out of all the traditional practices, Ayurveda, Yoga and Naturopathy, Unani, Siddha and Homeopathy are the official traditional systems of medicine which collectively provides healthcare to the vast majority of people of India and neighboring countries [31, 32]. Plants and plant-derived products are part of health care system since ancient human civilizations. The need of new chemical entities (NCEs) for health care is explored and served through the plant sources [33].

Previously we have studied several alternative medicines for their therapeutic potentials to alleviate allergic rhinitis in the allergic model rats [34 – 39]. Still, numerous numbers of plants remain unnoticed for their right medicinal uses due to lack of appropriate investigation and knowledge. On the other hand, efficacy of herbal medicines is well recognized by the system of alternative medicine, it is possible that these sources will have some better effective components which are expected to have lesser side effects and relatively safer compared to the existing modern medicines.

As a part of our ongoing search for anti-allergic compound(s) from natural sources, I have studied Ayurvedic plants having anti-allergic usage traditionally. Further experiments extended to the isolation of active compound(s) from plant extracts, synthesis of compounds, check the therapeutic effectiveness of compound(s) in in-vitro

and in-vivo allergic models. Additionally, mechanism of the compound(s) was also carried out to identify target protein for signaling mechanism in allergic rhinitis.

Section - 2

Plant screening for the exploration of anti-allergic activity.

2.1 Introduction:

Traditional medicines (TM) has been practiced worldwide for thousands of years and still remain as a good source of structurally important chemical substances that lead to the development of modern medicines [40, 41]. TM is very popular in Eastern Asia such as China, Korea and Japan and has been well investigated [42]. On the other hand, Ayurveda, a comprehensive and integral medicinal system, has been practiced in Indian subcontinent since 5000 BC [32, 33, 43 – 45].

Up-regulation of H1R has been observed in patients with allergic rhinitis [14, 46]. We have demonstrated that the level of H1R gene expression is firmly associated with the severity of allergic symptoms and compounds that suppress H1R gene up-regulation alleviate allergic symptoms [35, 39, 48 – 51].

Various oriental medicinal herbs have been reported to have anti-allergic activity both *in-vitro* and *in-vivo* [52 – 54]. Over the last few decades, extensive work has been done for the isolation and characterization of compounds derived from various plant species to uncover its chemical and biological properties [55, 56].

Again, plants substances for medication are believed to be less toxic compare to synthetic chemical compounds [57]. The secondary metabolites produces by the medicinal plants has unique attribution to use them as resources for pharmaceuticals, food additives and in fine chemicals [58].

2.2 Materials and Methods:

2.2.1 Materials:

PMA was purchased from Sigma-Aldrich (St. Louis, MI, USA). Minimal essential medium (MEM)-α was from Invitrogen (Carlsbad, CA, USA). RNAiso Plus

and the PrimeScript RT reagent Kit were from Takara Bio Inc. (Kyoto, Japan). Pre-developed TaqMan assay reagents of rat and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Applied Biosystems (Foster City, CA, USA). All other chemicals and solvents were of analytical grade.

2.2.2 Plant materials:

For the present study we have selected six plats (*Coccinia cordifolia, Moringa oleifera, Ocimum sanctum, Adhatora vasica, Tephrosia purpurea, Cinnamonnum tamala*) which have been reported to have anti-allergic usage in Ayurveda. Therefore, we have screened these plants for their significant anti-allergic properties based on their ability to suppress H1R mRNA in allergic model cells where HeLa cells used as a prototype for the study.

The specimens from the plant leaf, and or the entire aerial parts were collected after discussion with the traditional practitioner in Bangladesh. All the samples collected were authenticated by National Herbarium of Bangladesh. The collected plants materials were dried under shade for several days and ground to coarse powder. The powdered plant was extracted by maceration at room temperature using methanol (MeOH). The extract was concentrated in vacuo to afford methanolic extract.

2.2.3 HeLa cell culture:

HeLa cells were cultured at 37 °C under a humidified 5–95% CO_2-air atmosphere in MEM-α medium containing 8% FCS (Sigma-Aldrich) supplemented with 100 IU/ml penicillin (Sigma-Aldrich) and 50 μg/ml streptomycin (Sigma-Aldrich). HeLa cells were cultured to 70 -80% confluence in 35-mm dishes and were serum

starved for 24 h at 37 °C before treatment with 100 nM of PMA for 3 h. Cells were pretreated with indicated concentrations of the extracts for 1 h prior to PMA treatment.

2.2.4 Real-time quantitative RT-PCR:

After a 3-h treatment with PMA, the cells were harvested with 700 µl of RNAiso Plus, mixed with 140 µl of chloroform, and centrifuged at 15,000 rpm for 15 min at 4 °C. The aqueous phase was collected, and RNA was precipitated by the addition of isopropyl alcohol. After centrifugation at 15,000 rpm for 15 min at 4 °C, the resulting RNA pellet was washed with ice-cold 75% ethanol. Total RNA pellet was resolved in 10 µl of diethylpyrocarbonate (DEPC)-treated water, and 1 µg of each RNA samples was used for the reverse transcription reaction. RNA samples were reverse-transcribed to cDNA using a PrimeScript RT reagent Kit. TaqMan primers and the probe were designed using Primer Express software 490 (Applied Biosystems). Real-time PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems). The sequences of the primers and TaqMan probe were as follows: forward primer for human H1R, 5'-CAGAGGATCAGATGTTAGGTGATAGC-3'; reverse primer for human H1R, 5'-AGCGGAGCCTCTTCCAAGTAA-3'; TaqMan probe, FAM-CTTCTCTCGAACGGACTCAGATACCACC-TAMRA. To standardize the starting material, the human GAPDH gene (Applied Biosystems) was used, and data were expressed as the ratio of H1R mRNA to GAPDH mRNA.

2.2.5 Statistical analysis:

All data expressed as mean±SEM (n=3 – 4) of at least three independent experiments. Statistical analysis is carried out by unpaired student's t test. $P<0.05$ is considered statistically significant.

2.3 Results:

Stimulation of HeLa cells with PMA or histamine significantly increased H1R mRNA expression [59]. Therefore, we prepared different concentrations of plant samples for in-vitro study and checked their suppressive activity on PMA stimulated HeLa cells.

Among the six selected plants, four plants (*Moringa oleifera, Ocimum sanctum, Adhatora vasica, Tephrosia purpurea*) showed H1R gene suppression on PMA-induced up-regulation of H1R mRNA on HeLa cells (Fig: 2-1.). Other two plants (*Coccinia cordifolia, Cinnamonnum tamala*) did not exhibit any suppression of H1R mRNA.

Moringa oleifera dose dependently suppressed PMA stimulated H1R gene expression on HeLa cells. At a 400 µg/ml dose suppression goes below to the basal level. Although *Ocimum sanctum* and *Adhatora vasica* suppressed PMA-induced H1R mRNA dose dependently, but they presented less suppressive potency compared to *Moringa oleifera*. On the other hand, *Tephrosia purpurea* revealed highest suppressive effect on PMA-induced up-regulation of H1R mRNA on HeLa cells and at a dose of 50µg/ml, suppression goes below to the basal level.

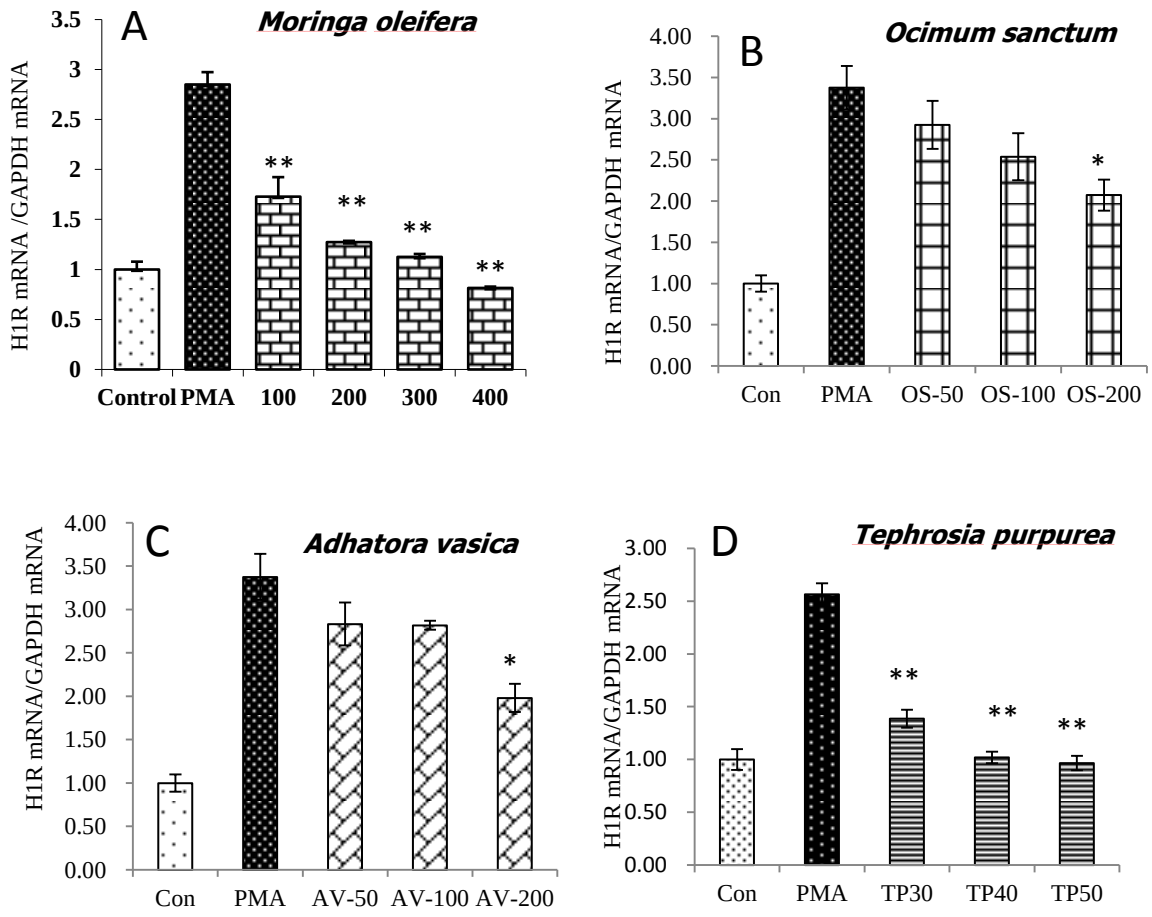


Figure: 2-1. Effects of methanolic extracts of Ayurvedic plants on PMA- induced up-regulation of H1R expression in HeLa cells.

HeLa cells were cultured to 70% confluence and serum starved for 24 h at 37°C. HeLa cells were preincubated with indicated concentrations of methanolic extract for 1 h before 100 nM PMA stimulation. H1R mRNA expression was determined using quantitative reverse transcription-polymerase chain reaction. Gene expression was measured after normalization with house-keeping gene GAPDH. (A) *Moringa oleifera,* (B) *Ocimum sanctum,* (C) *Adhatora vasica,* (D) *Tephrosia purpurea*. Data are expressed as means ± S.E.M.; $^{**}p < 0.01$, and $^{*}p < 0.05$ vs. PMA- treated cells ($n = 3–4$).

2.4 Discussion:

Up-regulation of H1R has been observed in patients with allergic rhinitis [14, 46]. We have demonstrated that the level of H1R gene expression is firmly associated with the severity of allergic symptoms and compounds that suppress H1R gene up-regulation alleviate allergic symptoms [35, 39, 47 – 51].

We previously demonstrated that H1R receptor gene is an allergic disease sensitive gene [60, 61] and compounds possessing the suppressive properties against elevation of H1R mRNA would be worth developing as anti-allergic drugs [35, 39, 47 – 51]. Histamine and phorbol 12-myristate-13-acetate (PMA) induced expression of H1R gene were well studied in HeLa cell and their mechanistic observation showed that such expression is resulted through PKC-δ dependent signaling [59, 62].

Methanolic extract of four plants (*Moringa oleifera, Ocimum sanctum, Adhatora vasica, Tephrosia purpurea*) exhibited H1R gene suppression on PMA-induced up-regulation of H1R mRNA on HeLa cells. Therefore, it can be evident that PKCδ – ERK signaling is involved in the suppression of H1R gene expression in-vitro. On the other hand, *Tephrosia purpurea* showed suppression to the basal level at low dose (50µg/ml) compared to other experimental plant extracts. Thus further study for the isolation of lead compound(s) from *Tephrosia purpurea* is worthwhile to carry on.

2.5 Conclusion:

We have chosen *Tephrosia purpurea* for the present study and attempted to isolate active compound(s) in a bioactivity guided manner.

Section -3

The isolation and synthesis of a novel benzofuran compound from *Tephrosia purpurea,* and the synthesis of several related derivatives, which suppress histamine H_1 receptor gene expression.

3.1 Introduction:

Allergic rhinitis (AR), also known as hay fever, affects 5–22% of the population globally [63] and its' causes are gradually being clarified [14, 46, 64, 65]. Histamine is a significant key mediator, and acts mainly through histamine H_1 receptor (H1R) [64, 65]. Determination of the amino acid sequence of H1R by two of the authors (H. F. and H. M.), [66] has been followed by research into AR therapies, [34, 35, 39 47 – 51] including the survey to identify suitable small molecule drugs.

Advances in biological and chemical technology have led to several modern medicines (mMeds) for palliative treatment of AR [67, 68]. Many mMeds are single compounds. Unfortunately, mMeds often cause clinically negative side effects, and no conclusive therapeutic agent for treating AR has yet been identified. In contrast, several traditionally developed medicines (tMeds) are mildly effective and produce few negative side effects [40, 41]. However, tMeds are generally complicated chemical mixtures and the scientific basis for their action is often unclear. Therefore, many researchers, including us, have been attempting to isolate and determine the structures of key compounds in these complicated mixtures, [55, 56] and examine their therapeutic utility for allergic diseases, including AR [38, 52 – 54, 69 – 72]. tMeds are generally well known in East Asian countries such as China, Japan and Korea [42, 73]. However, Indian subcontinent also directed our attention, because tMeds from Indian subcontinent may exhibit stronger anti-allergic activity than that at East Asian tMeds [33, 43 – 45].

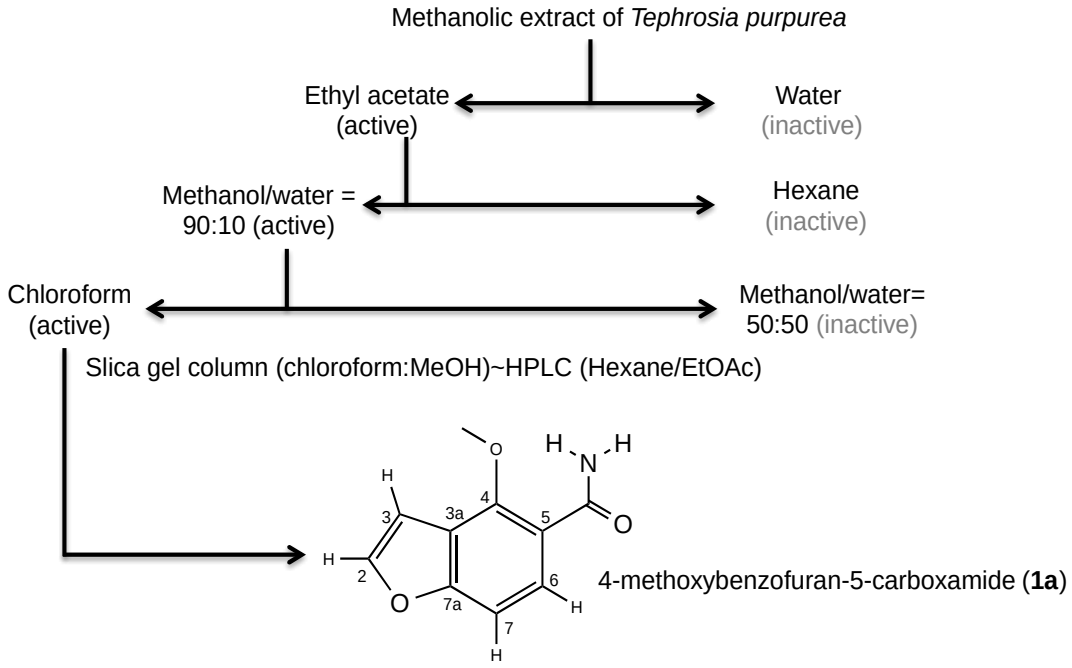
We report the isolation and synthesis of a new compound, 4-methoxybenzofuran-5-carboxamide (**1a**, Scheme 1), extracted from a plant, *Tephrosia*

purpurea. *T. purpurea* grows from east India to central Bangladesh. That is the first synthesis of **1a**; this report confirms the chemical structure of **1a** and its suppressive effect on H1R gene expression in allergic model cells [35, 39, 49, 51]. The synthesis of derivatives of **1a** and their suppressive effects are also demonstrated.

3.2 Results:

3.2.1 Isolation and its structural determination of 1a:

As shown in scheme 3-1., a methanolic extract of *Tephrosia purpurea* was fractionated using various solvents; the suppressive activity of each fraction was examined using phorbol 12-myristate 13-acetate (PMA)-induced up-regulation of H1R gene expression in HeLa cells using our previously established procedure [38, 69 – 72]. The chloroform fraction exhibited the highest activity; this fraction was further fractionated by silica gel column chromatography, followed by HPLC, resulting in the isolation of a single molecule. Extensive spectroscopic analysis identified the compound as 4-methoxybenzofuran-5-carboxamide (**1a**) [74 – 79]. Details of the structural determination are described below.



Scheme: 3-1. Extraction and isolation of 1a from *Tephrosia purpurea.*

Compound **1a** gave a pseudo-molecular ion peak at *m/z* 214.0498 (calcd for $C_{10}H_9NO_3Na$, 214.0480) on ESI-HRMS, suggesting the molecular formula is $C_{10}H_9NO_3$. The ^{1}H NMR spectrum showed a pair of *ortho*-coupled aromatic protons [δ_H 8.16 and 7.32 (each 1H, d, J = 8.8 Hz)], a pair of conjugated olefinic protons [δ_H 7.64 and 6.98 (each 1H, d, J = 2.2 Hz)], and a methoxyl signal (δ_H 4.19) (Table: 3-1.). The ^{1}H NMR spectrum also showed two broad D_2O exchangeable peaks at 7.78 and 5.72; these coupled with IR absorption bands at 3453 and 1654 cm^{-1}, implied the presence of primary carboxamide functionality. The ^{13}C NMR spectrum exhibited ten carbon signals, including a methyl, four sp^2 methines, four sp^2 quaternary carbons, and one carbonyl carbon.

The structure of **1a** was elucidated by 2D-NMR analysis. The olefinic proton signals at δ_H 7.64 (H-2) and 6.98 (H-3) resonated with the carbon signal at δ_C 144.9 and

105.2, respectively, in the HSQC spectrum. These resonances showed the HMBC correlations with the sp^2 quaternary carbon at δ_C 158.9 (C-7a) and 118.6 (C-3a) in each case, suggesting the presence of a furan moiety. In contrast, the following HMBC correlations indicated the presence of 4,5-disubstituted benzofuran structure; the aromatic proton signal at δ_H 7.32 (H-7) with C-3a; the carbon signals at δ_C 117.5 (C-5); the signal at δ_H 8.16 (H-6) with C-7a and the resonances at δ_C 152.9 (C-4). In addition, the positions of the carboxamide and the methoxy group were assigned to be C-5 and C-4, respectively, from the HMBC correlation of H-6 with the carbonyl carbon resonance and of the methoxy signal with C-4. Based on the spectral analysis described above, the structure of **1a** was assigned as 4-methoxybenzofuran-5-carboxamide.

Position	δ_H	Multiplicity & Coupling (Hz)	δ_C
2	7.64	d, *J* = 2.2	144.9 (CH)
3	6.98	dd, *J* = 0.8, 2.2	105.2 (CH)
4			152.9 (C)
5			117.5 (C)
6	8.16	d, *J* = 8.8	128.3 (CH)
7	7.32	d, *J* = 0.8, 8.8	107.1 (CH)
7a			158.9 (C)
3a			118.6 (C)
C=O			167.6 (C)
MeO	4.19	s	61.0 (CH_3)
NH	7.78	br	
NH	5.72	br	

Table: 3 - 1. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data for 1a.

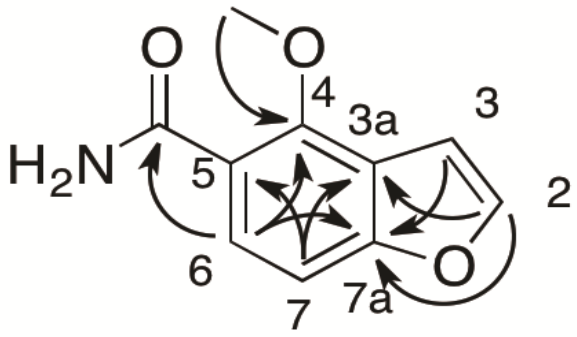
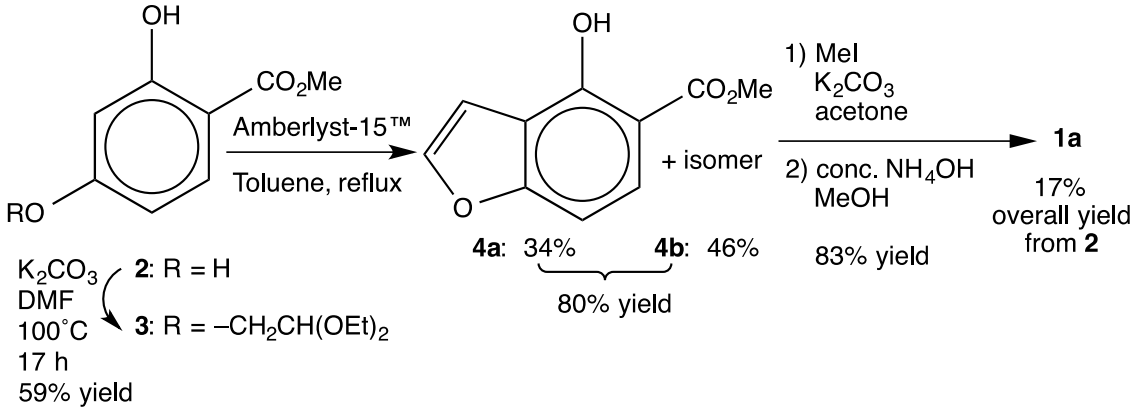


Fig: 3-1. HMBC correlation of the isolated compound.

3.2.2 Synthesis of 1a:

Compound **1a** was synthesized as shown in Scheme 3-2. Ether bond formation between two of the commercially available compounds, 2-bromoacetaldehyde diethyl acetal and methyl 2,4-hydroxybenzoate (**2**), was carried out in anhydrous DMF at 100˚C for 17 h in the presence of anhydrous potassium carbonate afforded **3** in 59% isolated yield. The phenoxide at the 4-position was more reactive than that at the 2-position, because the latter intra-molecularly has hydrogen bonds with the neighboring ester functionality. Treatment of **3** in toluene (0.07 mol/L) with Amberlyst-15, a polymer supported sulfonic acid, gave a mixture of benzofuran derivatives **4a** and **4b** in 80% combined yield. In contrast, use of a high concentration of **3** in toluene (0.70 mol/L) provided oligomer-like unidentified mixtures as major products, as expected from a previously reported preparation of benzofuran [80, 81]. It appeared that the concentration of **3** did not strongly influence the reaction rate of consumption of **3**, probably because the furan was produced intra-molecularly. On the other hand, over-reactions such as the dimerization of **4** and further oligomerizations are generally strongly inhibited under dilute conditions. Indeed, dilute conditions dramatically improved the chemical yield of **4**. Silica gel column chromatography easily separated

4a, an important synthetic intermediate for **1a**, from the corresponding isomer **4b**. The isolated yield of **4a** and **4b** was 34% and 46%, respectively.

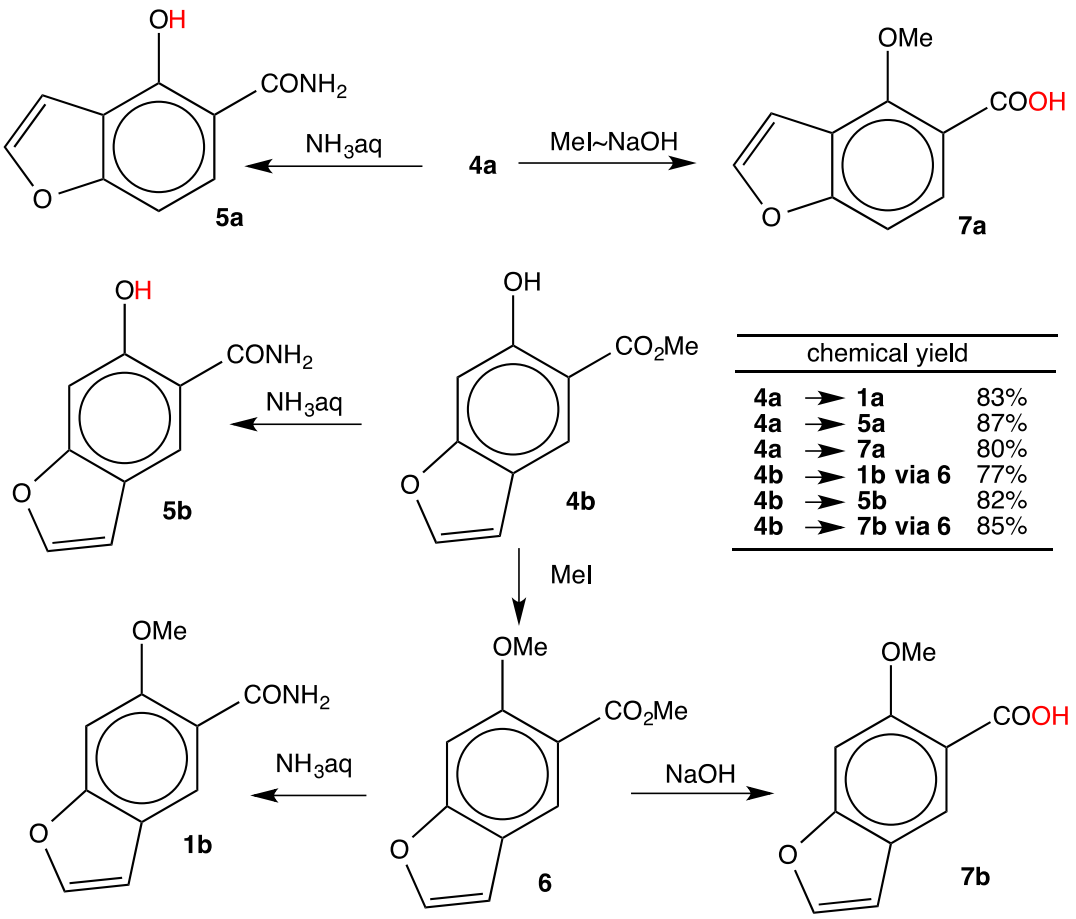


Scheme: 3 -2. Synthesis of 1a

Treatment of **4a** with iodomethane in the presence of potassium carbonate, followed by ammonolysis gave **1a** in 83% overall yield. All the analytical data of ^{1}H and ^{13}C NMR data from synthetic **1a** were identical with that of isolated **1a**, as were the IR and ESI-HRMS data. The suppressive effect of synthetic **1a** on H1R gene expression (*vide infra*) in allergic model cells was reproducibly as high as that of isolated **1a**.

3.3 Synthesis of the isomer and the related derivatives:

Treatment of **4a** with concentrated aqueous ammonia gave **5a** in 87% yield, and **4b** was similarly transformed to **5b** (82% yield). Compound **4a** was converted to **7a** via O-methylation and hydrolysis steps [82] in 80% yield, and **4b** was converted to **7b** (89% yield) in the same manner via **6**. Finally, the isomer **1b**, the sixth molecule in this small library, was synthesized from **6** in 80% yield via ammonolysis (Scheme: 3-3.).



chemical yield		
4a	→ **1a**	83%
4a	→ **5a**	87%
4a	→ **7a**	80%
4b	→ **1b via 6**	77%
4b	→ **5b**	82%
4b	→ **7b via 6**	85%

Scheme: 3 - 3. Synthesis of an isomer 1b and the related derivatives

3.4 Preliminary investigation of anti-allergic activity:

The activity of the six synthesized six compounds on PMA-induced H1R gene expression in HeLa cells was examined, [34, 35, 39, 47 – 51] and the results are summarized in Table 3-2. Isolated **1a** suppressed H1R mRNA up-regulation in HeLa cells with an IC_{50} of 75.3 µM (entry 1), and synthesized **1a** showed reproducible activity with an IC_{50} 83.7 µM (entry 2). It is noteworthy that the isomer **1b** suppressed H1R gene expression at a similar level, with an IC_{50} of 49.14 µM (entry3). Except **5b** (entry 5), the other modified derivatives were inactive.

Entry	Compound	Suppression (IC_{50}) (μM)
1	Isolated **1a**	75.3
2	Synthesized **1a**	83.7
3	**1b**	49.1
4	**5a**	–[a]
5	**5b**	84.6
6	**7a**	–[a]
7	**7b**	–[a]

a) No suppression of H1R mRNA expression was observed.

Table: 3-2. H1R mRNA suppression of the six compounds

3.5 Discussion:

Dawood, K. reported that certain functionalities such as –OH and –OMe on benzofuran skeleton contributed for several pharmacological effects [79]. Therefore, we synthesized derivatives containing –OH and –OMe functionalities.

Our interest is to the synthesis of library molecules using **1a** as a lead molecule. We believe that the primary carboxamide functionality should be fixed since carboxylic acids **7a** and **7b** were inactive (entries 6 and 7). In contrast, various substituents may be introduced on the phenoxy moiety since demethylated isomer **5b** showed the same level of activity as **1a** (entry 5). Figure 3-4. provides insights into the observed difference between **1a** and **1b**. Overlapping **1a** and **1b** in the orientation shown in (i) indicates that the relative position of the furan ring and carboxamide of **1a** is exactly the same as that of **1b**, whereas the position of the methoxy group of **1a** is quite different from that of **1b**. In contrast, the orientation shown in (ii) suggests that only the location of the furan

plane is different between the two compounds and that benzene ring and all the electronically negative elements overlap well.

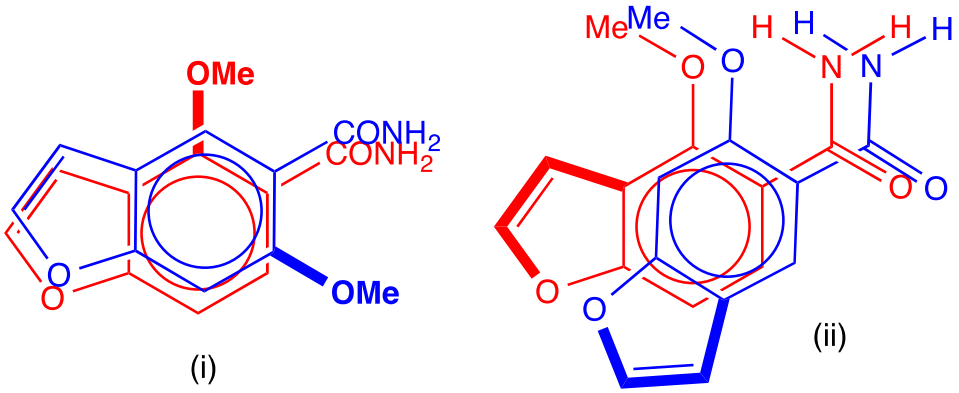
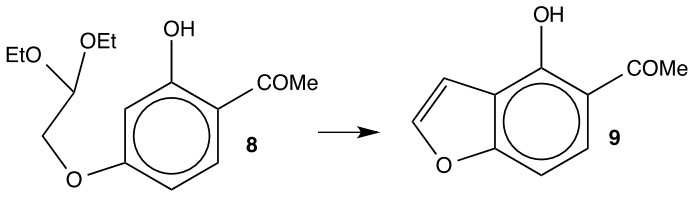


Figure: 3 -2. Overlapped structures of 1a and 1b.

During synthesis, at the beginning, we chose 0.7 mol/L as concentration of the reaction of **3**, due to the value reported in the following paper [80], where the reaction from ketone **8** to **9** was described. In the next related paper [81] same authors reported the dimerization reaction of **9**. Those results described both in references 14 and 15 confused us because the condition (including the concentration value) from **8** to **9** was almost same as the condition for dimerization of **9**. Therefore, we assumed that the value of the concentration in reference 17 was wrong. Accordingly, the step from **3** to **4a**/**4b** was easily improved by the high dilution condition (0.07 mol/L).



Although synthesis of **7a** was reported via different route [83], chemical yielded

yield is not satisfactory. Our synthetic scheme(s) is very reproducible with a satisfactory chemical yield. To understand anti-allergic activity of **1a,** we studied molecular mechanism of the compound as well [83].

3.6 Conclusion:

A novel compound, comprising a primary carboxamide on a benzofuran skeleton was isolated from a plant found in west India to central Bangladesh. The structure was determined by IR, ESI-HRMS, and NMR measurements. Preliminary examination of H1R mRNA suppression activity confirmed this compound to hold promise for the treatment of allergic diseases, including rhinitis. The chemical structure and the activity of the compound were confirmed by its total synthesis and two novel molecules designed from the isolated compound exhibited potential therapeutic utility. Although little of the lead compound can be isolated from natural source, the synthetic route described here will allow its synthesis on a scale of 10 g or more with high reproducibility. Our chemical synthesis and biological research efforts will continue in order to identify optimal drugs for treating allergies.

3.7 Experimental Sections:

IR spectra were recorded on a FT-IR 6200 spectrophotometer. ^{1}H NMR spectra were measured in chloroform-d_1 ($CDCl_3$) and referenced to tetramethylsilane using 400 MHz and 500 MHz spectrometers unless otherwise noted. ^{13}C NMR were measured in $CDCl_3$ and referenced to $CDCl_3$ (δ = 77.0) using 125 MHz spectrometers unless otherwise noted. Column chromatography was performed on silica gel (N-60). Thin layer chromatography as performed on pre-coated plates (0.25 mm, silica gel Kieselgel

60_{F254}). All the extract procedures were performed with commercially available organic solvents without further purification. All the chemical reactions were performed in oven-dried glassware equipped with calcium chloride tube. Reaction mixtures were stirred magnetically. Methanol (MeOH) was distilled over magnesium. N,N-dimethylformamide (DMF) was distilled over calcium hydride under reduced pressure. Toluene was distilled under normal pressure. Acetone was distilled over anhydrous potassium carbonate (K_2CO_3).

Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich. The pre-developed TaqMan Assay Reagent of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Applied Biosystems (Foster City, CA, USA). Minimal essential medium (MEM)-α was from Invitrogen (Carlsbad, CA, USA). RNAiso Plus and the PrimeScript RT reagent Kit were from Takara Bio Inc. (Kyoto, Japan). Methyl 2, 4-dihydroxy benzoate and bromoacetaldehyde diethyl acetal were purchased from Sigma-Aldrich and Tokyo Chemical Company (USA) respectively. All other chemicals and solvents were of analytical grade.

3.7.1 Plant Material:

Aerial parts of *Tephrosia purpurea* Pers. were collected in Bangladesh. The plant was identified by Dr. Sarder Nasir Uddin, National Herbarium Mirpur, Dhaka, Bangladesh (Accession No. 41236). The plant was then shed dried, grounded to obtained coarse powder material and stored until the present investigation.

3.7.2 Extraction and isolation:

The powdered plant (4.0 kg) was extracted by maceration at room temperature

using MeOH. The extract was concentrated in vacuo to afford a residue (118.8 g), which was dissolved in ethyl acetate (EtOAc), and washed with water. The organic layer was concentrated in vacuo to afford a residue (65.34 g), which was dissolved in MeOH:H_2O (90:10) and washed with hexane. The aqueous methanolic layer was concentrated in vacuo to afford a residue (35.0 g), which was dissolved in chloroform and washed with MeOH:H_2O=50:50. The chloroform layer was concentrated in vacuo to afford a residue (30 .0 g). Afterward, 16.0 g was subjected to silica gel column chromatography eluted with chloroform:MeOH (98.5:1.5, 97:3, 94:6, 90:10, 80:20, 1:1, and 0:1) to afford 24 fractions based on TLC examination. A part (30.0 mg) of fraction 9 (500.0 mg) was purified by HPLC (Cosmosil 5 SL, 4.6 mm id × 250 mm length) eluted with EtOAc:hexane (6:4, flow rate = 1.0 mL/min) in an isocratic condition to afford **1a** (6.0 mg). FT-IR (KBr): 3453, 2360, 1654, 1458, 1420, 1387, 1256, 1066, 978 cm^{-1}, ^1H NMR (500 MHz, $CDCl_3$) see Table 1; ^{13}C NMR (125 MHz, $CDCl_3$) see Table 1; HRESIMS m/z calcd for $C_{10}H_9NNaO_3$ $[M + Na]^+$ 214.0480, found: 214.0498.

3.7.3 Synthesis:

3.7.3.1 Methyl 4-hydroxy benzoate 2-acetaldehyde diethyl acetal (**3**):

To a solution of methyl 2,4-dihhydroxy benzoate (**2**) (1.00 g, 5.95 mmol) in DMF (10 mL), suspended with anhydrous potassium carbonate (K_2CO_3) (1.23 g, 8.93 mmol, 1.5 equiv.) with vigorously stirring, was added bromoacetaldehyde diethyl acetal (5.87 g, 29.8 mmol) drop wise over 5 min. The resulting suspension was heated at 100 ºC with stirring for 17 h. After being cooled, the suspension was poured into 5% of aqueous solution of potassium hydrogen sulfate ($KHSO_4$aq, 100 mL) at 0˚C, and extracted with EtOAc (120 mL × 3). The combined organic layers were washed with

brine, dried over anhydrous sodium sulfate (Na_2SO_4) and concentrated in vacuo. The residue was purified by silica gel column chromatography using hexane and EtOAc (8:2) to afford **3** as colorless oil (994.0 mg, 3.50 mmol, 59% yield). FT-IR (neat): 3145, 2977, 2884, 1917, 1672, 1624, 1584, 1506, 1442, 1374, 1350, 1257, 1225, 1195, 1178, 1136, 1072, 980, 958, 886, 855, 780, 733, 697, 655 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 10.94 (s, 1H, OH), 7.73 (d, J = 9.2 Hz, 1H), 6.48–6.45 (m, 2H), 4.83 (t, J = 5.2 Hz, 1H), 4.02 (d, J = 5.2 Hz, 2H), 3.91 (s, 3H), 3.76 (dq, J = 6.8, 9.4 Hz, 2H), 3.63 (dq, J = 6.8, 9.4 Hz, 2H), 1.25 (t, J = 6.8 Hz, CH_3 × 2); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.4 (C), 164.5 (C), 163.7 (C), 131.3 (CH), 107.8 (CH), 105.8 (C), 101.5 (CH), 100.2 (CH), 68.6 (CH_2), 62.8 (CH_2), 52.0 (CH_3), 15.4 (CH_3); HRESIMS m/z calcd for $C_{14}H_{19}O_6$ $[M - H]^+$ 283.1182, found: 283.1172.

3.7.3.2 Methyl 4-hydroxybenzofuran-5-carboxylate (4a) and methyl 6-hydroxybenzofuran -5-carboxylate (4b):

A solution of **3** (389.5 mg, 1.37 mmol) in toluene (20 mL) suspended with Amberlyst-15® (51.0 mg) was stirred at reflux for 4 h, equipped with dean-stark apparatus to remove water. After being cooled, the resulting suspension was filtered, and the residue was washed with toluene (80 mL). The combined filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography eluted with EtOAc/hexane (1:9) to afford **4a** (89.5 mg, 0.47 mmol; 34% yield) and **4b** (121.1 mg, 0.63 mmol, 46% yield), respectively.

4a: FT-IR (KBr): 3414, 3149, 3125, 3029, 2971, 2859, 1868, 1678, 1629, 1471, 1445, 1357, 1284, 1235, 1195, 1170, 1133, 1050, 982, 748 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 11.47 (s, 1H, OH), 7.78 (d, J= 8.8 Hz, 1H), 7.57 (d, J=2.0, 1H), 7.04 (d, J=8.8 Hz,

1H), 6.98 (d, J=2.0, 1H), 3.97 (s, 3H); ^{13}C NMR (125 MHz $CDCl_3$) δ 171.2 (C), 159.6 (C), 157.5 (C), 144.4 (CH), 126.0 (CH), 117.1 (C), 106.0 (C), 104.8 (CH), 103.9 (CH), 52.3 (CH_3); HRESIMS m/z calcd for $C_{10}H_8NaO_4$ $[M + Na]^+$ 215.0320, found: 215.0322. **4b**: FT-IR (KBr): 3413, 3120, 1663, 1557, 1443, 1414, 1369, 1286, 1258, 1212, 1186, 1143, 1072, 1013, 948, 845, 790 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.92 (s, 1H, OH), 8.13 (s, 1H), 7.54 (d, J=2.4 Hz, 1H), 7.07 (s, 1H), 6.70 (d, J = 2.4 Hz, 1H), 3.98 (s, 3H); ^{13}C (125 MHz, $CDCl_3$): δ 170.9 (C), 159.8 (C), 159.4 (C), 145.5 (CH), 123.1 (CH), 120.3 (C), 109.2 (C), 106.6 (CH), 99.4 (CH), 52.4 (CH_3); HRESIMS m/z calcd for $C_{10}H_7O_4$ $[M – H]^+$ 191.0350, found: 191.0351.

3.7.3.3 4-Hydroxybenzofuran-5-carboxamide (5a):

To a solution of **4a** (90.0 mg, 0.468 mmol) in MeOH (2.0 mL) was added an aqueous solution of conc. Ammonia (NH_3aq) (28%, 10 mL). The resulting mixture was stirred at 36º C for 24 h, and concentrated in vacuo. The residue was poured into HClaq (1N, 5.0 mL) and extracted with EtOAc (50mL × 3). The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography eluted with chloroform/MeOH (9:1) to afford **5a** (72.0 mg, 0.41 mmol, 87% yield). FT-IR (KBr): 3552, 3407, 3179, 1611, 1531, 1471, 1423, 1390, 1309, 1265, 1226, 1201, 1131, 1090, 1049, 1018, 941, 878, 754 cm^{-1}; ^{1}H NMR (500MHz, DMSO-d_6) δ 14.28 (s, 1H, OH), 8.45 (brs, 1H, NH), 7.96 (d, J = 2.0 Hz, 1H), 7.93 (brs, 1H, NH), 7.81 (d, J = 8.5 Hz, 1H), 7.13 (d, J = 8.5 Hz, 1H), 7.03 (d, J = 2.0 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 173.1 (C), 157.8 (C), 157.3 (C), 145.3 (CH), 124.2 (CH), 116.8 (C), 107.4 (C), 104.6 (CH), 102.6 (CH); HRESIMS m/z calcd for $C_9H_7NO_3$ $[M]^+$: 177.0426, found: 177.0424.

3.7.3.4 6-Hydroxybenzofuran-5-carboxamide (5b):

To a solution of **4b** (97.6 mg, 0.508 mmol) in MeOH (2.0 mL) was added NH_3aq (28%, 10 mL). The resulting mixture was stirred at 36º C for 5 h, and concentrated in vacuo. The residue was poured into HClaq (1N, 5.0 mL) and extracted with EtOAc (50 mL × 3). The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography eluted with chloroform/MeOH (9:1) to afford **5b** (74.0 mg, 0.418 mmol, 82% yield). FR-IR (KBr): 3547, 3422, 3195, 1666, 1638, 1605, 1543, 1466, 1400, 1284, 1236, 1189, 1150, 1114, 1060, 1016, 832 cm^{-1}; ^1H NMR (400 MHz, DMDO-d_6) δ 13.26 (s, 1H, OH), 8.48 (brs, 1H, NH), 8.18 (s, 1H), 7.90 (brs, 1H, NH), 7.90 (d, J = 2.0 Hz, 1H), 7.04 (s, 1H,), 6.90 (d, J = 2.0 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.6 (C), 159.6 (C), 157.5 (C), 145.9 (CH), 120.9 (CH), 119.0 (C), 111.2 (C), 106.7 (CH), 98.8 (CH); HRESIMS m/z calcd for $C_9H_7NNaO_3$ $[M + Na]^+$: 200.0324, found: 200.0336.

3.7.3.5 Methyl 6-methoxybenzofuran-5-carboxylate (6):

A mixture of **4b** (486mg, 2.529 mmol), K_2CO_3 (1.818g, 13.15 mmol), iodomethane (2.59g, 25.29 mmol) in acetone (25 mL) was refluxed for 5 h. After being cooled, the resulting suspension was filtered. The filtrate was concentrated in vacuo. The residue was diluted with ether (50 mL), and the resulting suspension was filtered. The filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (Hexane/EtOAc, 7:3) to afford **6**, a pale yellow oil (500 mg, 2.425mmol, 96% yield). FT-IR (neat): 3107, 2951, 2840, 1720, 1590, 1538, 1475, 1433, 1353, 1298, 1253, 1199, 1177, 1137, 1070, 1008, 899, 834 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.07 (s, 1H), 7.57 (d, J = 2.4 Hz, 1H), 7.10 (s, 1H), 6.73 (d, J = 2.4 Hz), 3.95

(s, 3H), 3.91 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.8 (C), 158.0 (C), 157.8 (C), 145.2 (CH), 124.8 (CH), 120.0 (C), 116.6 (C), 106.7 (CH), 95.4 (CH), 56.4 (CH_3), 52.1 (CH_3); HRESIMS m/z calcd for $C_{11}H_{10}NaO_4$ $[M + Na]^+$: 229.0477, found: 229.0473.

3.7.3.6 4-Methoxybenzofuran-5-carboxylic acid (7a):

A solution of **4a** (200.0 mg, 1.04 mmol) in acetone (11 mL) were added K_2CO_3 (747.0 mg, 5.41 mmol) and iodomethane (1.47 g, 10.4 mmol), and the resulting suspension was stirred at reflux for 3 h. After being cooled to room temperature, the resulting suspension was filtered. The filtrate was concentrated in vacuo, and diluted with ether (30 mL). The resulting suspension was again filtered, and the filtrate was concentrated in vacuo. The residue was used in the next step without further purification.

To a solution of the residue in MeOH (4.0 mL) was added an aqueous solution of sodium hydroxide (NaOHaq) (3N, 3.0 mL). The mixture was stirred for 7 h at room temperature, poured into HClaq (2N, 15 mL), extracted with EtOAc (40 mL × 3). The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography eluted with chloroform/methanol (9:1) to afford **7a** (160mg, 0.833 mmol, 80% yield); FT-IR (KBr): 3413, 3138, 2993, 1693, 1617, 1550, 1472, 1427, 1417, 1366, 1339, 1285, 1261, 1239, 1176, 1076, 987, 924, 750 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.93 (s, 1H), 8.15 (d, J = 8.8 Hz, 1H), 7.69 (d, J = 2.4 Hz, 1H), 7.36 (d, J = 8.8 Hz, 1H), 7.02 (d, J = 2.4 Hz, 1H), 4.34 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.0 (C), 159.8 (C), 153.4 (C), 145.6 (CH), 129.4 (CH), 117.8 (C), 113.9 (C), 107.9 (CH) 105.2 (CH), 61.6 (CH_3); HRESIMS m/z calcd for $C_{10}H_8NaO_4$ $[M + Na]^+$: 215.0320, found: 215.0321.

3.7.3.7 6-Methoxybenzofuran-5-carboxylic acid (7b):

To a solution of **6** (360.5 mg, 1.75 mmol) in MeOH (5.0 mL) was added NaOHaq (3N, 3.0 mL). The mixture was stirred for 5 h at room temperature, poured into HClaq (2N, 15 mL), and extracted with EtOAc (40 mL × 3). The combined organic layers were washed with brine (60 mL), dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography eluted with chloroform/methanol (9:1) to afford **7b** (300.0 mg, 1.56 mmol, 89% yield). FT-IR (KBr): 3413, 3235, 1690, 1638, 1618, 1581, 1540, 1475, 1427, 1406, 1356, 1270, 1209, 1188, 1146, 1066, 1003, 901, 828 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 10.85 (s, 1H, COOH), 8.49 (s, 1H), 7.64 (d, J = 2.4 Hz, 1H), 7.19 (s, 1H, 6.81 (d, J = 2.4 Hz, 1H), 4.13 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.7 (C), 158.4 (C), 156.3 (C), 146.2 (CH), 127.3 (CH), 122.0 (C), 113.9 (C), 107.0 (CH), 95.3 (CH), 57.1 (CH_3); HRESIMS m/z calcd for $C_{10}H_8NaO_4$ $[M + Na]^+$: 215.0320, found: 215.0312.

3.7.3.8 4-Methoxybenzofuran 5-carboxamide (synthetic **1a**):

A solution of **4a** (200 mg, 1.04 mmol) in acetone (11 mL) were added K_2CO_3 (747.4 mg, 5.41 mmol) and iodomethane (1.48 g, 10.43 mol), and the resulting suspension was stirred at reflux for 3 h. After being cooled to room temperature, the resulting suspension was filtered. The filtrate was concentrated in vacuo, and diluted with ether (30 mL). The resulting suspension was again filtered, and the filtrate was concentrated in vacuo. The residue was used in the next step without further purification.

To a solution of the residue in MeOH (5.0 mL) was added NH_3aq (40 mL, 28%), and the mixture was stirred at 35 ºC for 27 h. The resulting solution was concentrated in

vacuo. To the residue was added HClaq (1N, 10 mL), extracted with EtOAc (60 mL × 3). The combined organic layers were washed with a saturated aqueous solution of sodium hydrogen carbonate (50 mL), brine (20 mL), dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel chromatography eluted with chloroform/methanol (9:1) to afford **1a** (165.0 mg, 0.863 mmol, 83% yield). FT-IR (KBr): 3459, 3162, 1668, 1605, 1579, 1475, 1461, 1419, 1387, 1348, 1322, 1251, 1233, 1067, 975, 742 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.16 (d, J = 8.8 Hz, 1H), 7.77 (brs, 1H, NH), 7.63 (d, J = 2.2 Hz, 1H), 7.31 (d, J = 8.8 Hz, 1H), 6.98 (d, J = 2.2 Hz, 1H), 5.76 (brs, 1H, NH), 4.19 (s, 3H); ^{13}C (125 MHz, $CDCl_3$) δ 167.3 (C), 158.9 (C), 152.8 (C), 145.0 (CH), 128.4 (CH), 118.6 (C), 117.6 (C), 107.2 (CH), 105.3 (CH), 61.2 (CH_3); HRESIMS m/z calcd for $C_{10}H_9NNaO_3$ $[M + Na]^+$ 214.0480, found: 214.0485.

3.7.3.9 Synthesis of 4-Methoxybenzofuran 5-carboxamide (1a) on a large scale from 2:

Two times of the large-scale examinations of the three steps (from **2** to **3**, from **3** to **4a**, and from **4a** to **1a**) confirmed that the overall yield from **2** to **1a** was reproducible (17% ± 5%, 0.59 × 0.34 × 0.83 = 0.17). The following results were the one of the two examinations. Compound **2** (56.7 g, 336.9 mmol) was converted to **3** (56.5 g, 198.8 mmol) in 59% yield. Compound **4a** (13.0 g, 37.6 mmol) was obtained from **3** (198.8 mmol) in 34% yield along with **4b**. Compound **1a** (10.72 g, 56.1 mmol) was afforded from **4a** (37.6 mmol) in 83% yield.

3.7.3.10 6-Methoxybenzofuran-5-carboxamide (1b):

To a solution of **6** (502 mg, 2.435 mmol) in MeOH (7.0 mL) was added NH_3aq (28%, 30 mL). The mixture was stirred at 35ºC for 12 h, concentrated in vacuo, poured into HClaq (1N, 15 mL) at 0˚C, and extracted with EtOAc (100 mL × 3). The combined organic layers were washed with NaOHaq (1N, 100 mL), brine (150 mL), dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel chromatography eluted with chloroform/MeOH (9:1) to afford **1b** (373.0 mg, 1.95 mmol, 80% yield). FR-IR (KBr): 3404, 3179, 1643, 1603, 1538, 1475, 1435, 1376, 1288, 1201, 1154, 1067, 1003, 905, 826 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (s, 1H), 7.59 (d, J = 2.4 Hz , 1H), 7.77 (brs, 1H, NH), 5.79 (brs, 1H, NH), 7.12 (s, 1H), 6.78 (d, J = 2.4 Hz, 1H), 4.02 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.4 (C), 157.7 (C), 156.4 (C), 145.3 (CH), 125.7 (CH), 121.0 (C), 117.5 (C), 107.0 (CH), 94.8 (CH), 56.4 (CH_3); HRESIMS m/z calcd for $C_{10}H_9NNaO_3$ $[M + Na]^+$: 214.0480, found: 214.0512.

3.7.4 ***HeLa cell experiments:***

HeLa cells were cultured at 37 °C under a humidified (5% CO_2, 95% air) atmosphere in MEM-α medium containing 8% FCS (Sigma-Aldrich) supplemented with 100 IU/ml penicillin (Sigma-Aldrich) and 50 μg/ml streptomycin (Sigma-Aldrich). HeLa cells were cultured to 70% confluence in 35-mm dishes and were serum starved for 24 h at 37 °C before treatment with 100 nM of phorbol-12-myristate-13-acetate (PMA) for 3 h. Cells were pretreated with TP extract, its different fractions, isolated compound as well as synthesized compounds for 1 h prior to PMA stimulation.

3.7.5 *Real-time quantitative RT-PCR*:

After a 3-h treatment with PMA, the cells were harvested with 700 μl of RNAiso Plus (Takara Bio Inc.), mixed with 140 μL of chloroform, and centrifuged at 17,400 × *g* for 15 min at 4 °C. The aqueous phase was collected, and RNA was precipitated by the addition of isopropyl alcohol. After centrifugation at 17,400 × *g* for 15 min at 4 °C, the resulting RNA pellet was washed with ice-cold 75% ethanol. Total RNA pellet was resolved in 10 μl of diethylpyrocarbonate-treated water, and 1 μg of each RNA samples was used for the reverse transcription reaction. RNA samples were reverse-transcribed to cDNA using a PrimeScript RT reagent Kit. TaqMan primers and the probe were designed using Primer Express software (Applied Biosystems). Real-time PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems). The sequences of the primers and TaqMan probe were as follows: forward primer for human H1R, 5′-CAGAGGATCAGATGTTAGGTGATAGC-3′; reverse primer for human H1R, 5′-AGCGGAGCCTCTTCCAAGTAA-3′; TaqMan probe, FAM-CTTCTCTCGAACGGACTCAGATACCACC-TAMRA. To standardize the starting material, the human GAPDH gene (Applied Biosystems) was used, and data were expressed as the ratio of H1R mRNA to GAPDH mRNA.

Section -4

A novel benzofuran, 4-methoxybenzofuran-5-carboxamide, from *Tephrosia purpurea* suppressed histamine H_1 receptor gene expression through a protein kinase C-δ-dependent signaling pathway.

4.1 Introduction

Allergic rhinitis (AR), a common allergic disease, affects 5%–22% of the global population [63]. Histamine is the key mediator and plays pivotal roles in allergic diseases, mainly following binding to the histamine H_1 receptor (H1R) [64, 65]. Toluene-2,4-diisocyanate (TDI) is a leading cause of asthma in industrialized countries [84], and repeated intranasal application of TDI caused release of histamine from mast cells and led to the development of nasal hypersensitivity in guinea pigs and rats [85-87]. Upregulation of H1R has also been observed in patients with AR [14, 46]. Accordingly, we demonstrated that H1R mRNA expression was strongly correlated with the severity of allergic symptoms in TDI-sensitized allergic model rats and in patients with pollinosis [47, 48]. Moreover, compounds that suppress the upregulation of H1R gene expression improved allergic symptoms [34, 35, 47 – 50]. Although advances in biological and chemical technologies have led to several modern palliative treatments for AR [67, 68], no therapeutic agents have yet been conclusively identified.

Helper T cell type 2 (Th2) cytokines including interleukin (IL)-4, IL-5, IL-9, and IL-13 reportedly play substantial roles in the pathogenesis of allergic inflammation [88], and increasing experimental evidence suggests the importance of histamine–cytokine networks in allergic inflammation [22–24]. Recently we showed that histamine and phorbol 12-myristate 13-acetate (PMA) upregulated H1R expression at both mRNA and protein levels by activating protein kinase C-δ (PKCδ)/extracellular signal-regulated kinase (ERK)/poly(ADP-ribose) polymerase-1 (PARP-1) signaling in HeLa cells expressing H1R endogenously [59, 62]. Moreover, stimulation with histamine- or PMA-induced phosphorylation of PKCδ at Tyr^{311} led to the translocation of PKCδ from the cytosol to the Golgi, where ERK was phosphorylated by activated PKCδ in response

to histamine or PMA [62].

Tephrosia purpurea (L.) Pers. is a perennial plant that belongs to the family Fabaceae and grows in Bangladesh, India, Sri Lanka, and various other tropical countries. This plant has been traditionally used for the treatment of asthma, rheumatism, chronic diarrhea, cirrhosis, and urinary disorders [89]. Phytochemical investigations showed *T. purpurea* contained rotenoids, flavanones, isoflavones, and sterols [90–92]. In addition, extracts from *T. purpurea* reportedly showed various pharmacological activities, including anti-inflammatory, antiulcer, antimicrobial, anti-allergic, anti-diabetic, and antitumor activities [90, 93–98]. In our previous study, we isolated the novel benzofuran, 4-methoxybenzofuran-5-carboxamide (MBCA), from *T. purpurea* and reported its chemical synthesis [99]. We also showed that MBCA suppressed PMA-induced upregulation of H1R mRNA in HeLa cells. Previous studies showed that benzofuran compounds possessed various biological activities, including anti-allergy and anti-inflammatory activities that ameliorate asthma, rheumatism, and ulcers [74–78]. However, the underlying mechanisms behind these activities remain unknown.

In the present study, we investigated the molecular mechanism of suppressive effect of MBCA on PMA-induced upregulation of H1R gene expression in HeLa cells. We also investigated *in vivo* anti-allergic effects of MBCA in TDI-sensitized rats. The present data suggest that MBCA alleviates nasal symptoms in TDI-sensitized rats by inhibiting H1R and Th2 cytokine expression. The molecular mechanism underlying its suppressive effect for H1R gene expression is mediated by the inhibition of PKCδ activation.

4.2. Materials and Methods

4.2.1. Materials:

PMA, furosemide, carbamazepine, and verapamil were purchased from Sigma–Aldrich (St. Louis, MI, USA). NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Rat, gerbil, dog, pig, and bovine liver microsomes were obtained from Xenotech (Lenexa, KS, USA). Caco-2 cells were purchased from ATCC (Manassas, VA, USA), and apical and basal culture plates were purchased from BD (Bedford, MA, USA). Minimal essential medium (MEM)-α was purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Gibco BRL Life Technology (Grand Island, NY, USA). RNAiso Plus and the PrimeScript RT reagent Kits were purchased from Takara Bio Inc. (Kyoto, Japan). TDI was purchased from Wako Pure Chemical (Osaka, Japan). RNA later and pre-developed TaqMan assay reagents for rodent and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Applied Biosystems (Foster City, CA, USA). All other chemicals and solvents were of analytical grade.

4.2.2. Preparation and use of MBCA for in vitro and in vivo study:

MCBA (Fig. 4-1.) was synthesized as described previously [34]. For *in vitro* studies using HeLa cells, MBCA was initially dissolved in DMSO followed by dilution to indicated concentration(s) in MEM-α medium. For *in vivo* studies using TDI-sensitized rats, MBCA was dissolved in DMSO followed by suspension at indicated concentrations in 0.5% carboxymethyl cellulose vehicle solution. Doses of 15, 25, and 35 mg/kg/day were administered orally at 1 h before and 3 h after TDI stimulation

according to pharmacokinetic properties.

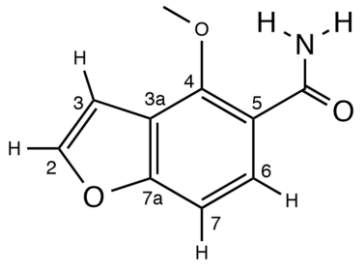


Figure: 4-1. Structure of 4-methoxybenzofuran-5-carboxamide (MBCA).

4.2.3. Cell culture:

HeLa cells were cultured at 37°C in a humidified incubator containing 5% and 95% CO_2 and atmospheric air, respectively, in MEM-α medium containing 8% FCS (Sigma–Aldrich) supplemented with 100 IU/ml penicillin (Sigma–Aldrich) and 50 μg/ml streptomycin (Sigma–Aldrich). HeLa cells were cultured to 70% confluence in 35-mm dishes and were serum starved for 24 h at 37°C before treatment with 100 nM PMA or 100 μM histamine for 3 h. Cells were pretreated with indicated concentrations of MBCA for 1 h prior to PMA or histamine treatments. RBL-2H3 cells were cultured in MEM containing 10% fetal bovine serum, 120 IU/ml penicillin, and 120 μg/ml streptomycin.

4.2.4 In vitro pharmacokinetic studies using liver microsomes:

MBCA was incubated for 0, 5, 10, 20, 30, and 60 min with rat, gerbil, dog, pig, and bovine liver microsomes in phosphate buffer (pH 7.4) in a temperature-controlled water bath at 37°C. And a matrix blank without test compound was used as a control. The final concentration of MBCA in 100 μl reaction mixtures was 1 μM, and the

mixture contained liver microsomes (0.5 mg/ml), NADP (1.3 mM), glucose-6-phosphate (3.3 mM), glucose-6-phosphate dehydrogenase (0.4 U/ml), and $MgCl_2$ (3.3 mM). Enzyme reactions were started by the addition of a NADP regenerating system comprising NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and $MgCl_2$ into 96 deep well plates and were performed for the stated times. Reactions were terminated by addition of 300 µl of ice cold acetonitrile, and reaction mixtures were then mixed and centrifuged at 4,000 rpm for 20 min at 15°C. Supernatants (120 µl) were diluted with 120 µl of water, and 25 µl aliquots were injected into an LC–MS/MS instrument. Interspecies enzyme kinetics studies were performed using the same reagents and procedures, with the exception of liver microsomes, which are highly species specific.

4.2.5 Caco-2 experimental procedure:

For MBCA permeability assays, human colon carcinoma (Caco-2) cells were seeded at 6.3×10^4 cells/cm^2 and were grown in DMEM containing 4.5 g/L glucose, 10% (v/v) fetal bovine serum, 1% (v/v) glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), and 1% (v/v) MEM nonessential amino acids. Cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO_2 with controlled humidity, and media were changed every 2–3 days. Routine passaging of all cell stocks was performed in 75-cm^2 culture flasks. Studies were conducted on Caco-2 cells at passages of 25–30, and 75 µl aliquots of cell suspensions containing 2.5×10^5 cells/ml were transferred into apical wells with 40 µl of media in feeder trays. Plates were incubated at 37°C in 5% CO_2 with controlled humidity, and Caco-2 cells were grown for 21 days. Culture media was replaced at 40–72 h after seeding, and every second day thereafter. For apical to

basolateral permeability assays, apical wells were washed with buffer at pH 6.5, and basal wells were washed with phosphate buffer at pH 7.4. Subsequently, 250 µl phosphate buffer (pH 7.4) was added to basal wells of transport analysis plates, and 75 µl aliquots of MBCA, furosemide, carbamazepine, or verapamil solutions (2 µM) in phosphate buffer (pH 6.5) were added to apical wells.

For basolateral to apical permeability assays, aliquots (25 µl) of cell suspensions containing 7.5×10^5 cells per ml were transferred to the bottom sides (keeping the plate upside down) of apical wells, and plates were incubated upside down at 37°C in 5% CO_2 with controlled humidity for 2 h. Subsequently, plates were inverted to the correct side up, and 75 µl of media was added to apical wells, and 40 µl media was added to feeder trays. Apical wells were washed with phosphate buffer at pH 7.4, and basal wells were washed with phosphate buffer at pH 6.5. Subsequently, 250 µl of phosphate buffer (pH 6.5) was added to the wells of basal plates (Transport Analysis Plate), and 75 µl aliquots of MBCA, furosemide, carbamazepine, or verapamil solutions (2 µM) in phosphate buffer (pH 7.4) were added to the apical wells. Apical plates were then placed onto basal plates, and lids were used to prevent evaporation. Plate assemblies were incubated at 37°C for 2.5 h (without shaking) under 5% CO_2 and 95% relative humidity (RH). Apical plates were then separated from basal plates, and aliquots from acceptor wells were taken, diluted, and quantified using LC–MS/MS.

To optimize the membrane integrity of Caco-2 monolayers, trans-epithelial electrical resistance (TEER) was determined during cell culture. Significant increases in TEER were observed between days 15 and 20. An additional membrane integrity test was performed after Caco-2 cell experiments using lucifer yellow (LY) and fluorimetry. Solutions in apical wells were discarded by inverting the plate on tissue paper.

Phosphate buffer (pH 7.4; 250 µl) was then added to the wells of basal plates and 75 µl of LY (0.1 mg/ml) in phosphate buffer (pH 7.4) was added to the wells of apical plates. Apical plates were then placed on to basal plates, and plate assemblies were incubated at 37°C for 1 h under 5% CO_2 and 95% RH. Apical plates were then separated from basal plates, and 100 µl aliquots of basal well solutions were transferred to cuvettes for measurements of fluorescence (Ex: 432 nm, Em: 530 nm). The fluorescence of 100 µl of phosphate buffer (pH 7.4) only and 100 µl of LY (0.1 mg/ml) only was measured as controls.

4.2.6. Animal study:

Six-week-old male Brown Norway rats (200–250 g; SLC, Hamamatsu, Japan) were allowed free access to water and food and were kept in a room at 25°C ± 2°C and 55% ± 10% humidity with a 12-h light–dark cycle. Rats were divided into three groups of four rats each, including a control group, a TDI-sensitized group, and a test group. All procedures involving animals were conducted in accordance with the Guidelines for Animal Experiments and were approved by the Ethical Committee for Animal Studies of the School of Medicine, Tokushima University (protocol number 14055).

Sensitization with TDI was performed according to previously described methods [36] with slight modifications (Fig. 4-2). Briefly, 10 µl aliquots of 10% TDI solution in ethyl acetate were applied bilaterally to nasal vestibules of each rat once daily for five consecutive days. The sensitization procedure was then repeated after a 2-day interval, and on the ninth day after the second sensitization, 10 µl of 10% TDI solution was again applied to the nasal vestibule to provoke nasal symptoms. The control group was sensitized and provoked with 10 µl of ethyl acetate only using the

same procedure. Nasal symptoms were measured according to numbers of sneezes and the extent of watery rhinorrhea using previously described methods [85]. After TDI provocation, animals were placed in a cage (one animal per cage) and numbers of sneezes and severity of watery rhinorrhea were examined for 10 min. The extent of watery rhinorrhea was estimated in the range of 0–3 according to the criteria presented in Table 4-1.

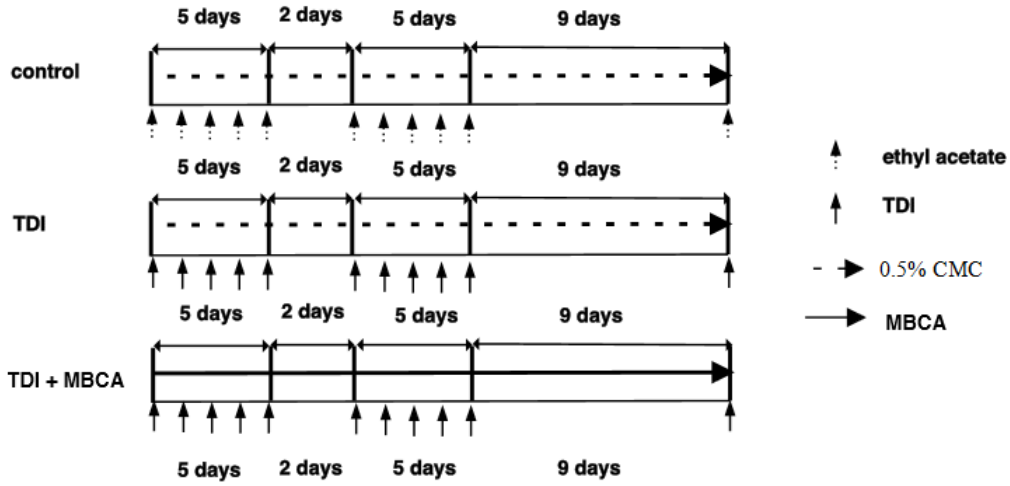


Figure: 4-2. Experimental protocol.

Rats were sensitized by intranasal application of 10 µl of 10% TDI in ethyl acetate for 2 weeks. After 1 week, nasal symptoms were further provoked using 10 µl of 10% TDI. Control rats were sensitized with ethyl acetate only. MBCA was orally administered at 15, 25, and 35 mg/kg/day twice daily as described in the Materials and Methods section.

Nasal response	Score			
	0	1	2	3
Watery rhinorrhea	(–)	At the nostril	Between 1 and 3	Drops of discharge from nose
Swelling and redness	(–)	Slightly swollen	Between 1 and 3	Strong swelling and redness

Table 4-1. Criteria for grading the severity of TDI-induced nasal responses in rats.

4.2.7. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR):

After 3-h treatment with PMA or histamine (for HeLa cells) the cells were harvested in 700 μl of RNAiso Plus and were mixed with 140 μl of chloroform and centrifuged at 15,000 rpm for 15 min at 4°C. Subsequently, the aqueous phase was collected, and RNA was precipitated by adding isopropyl alcohol. After centrifugation at 15,000 rpm for 15 min at 4°C, total RNA pellets were washed with ice-cold 75% ethanol, and the pellets were resuspended in 10 μl of diethylpyrocarbonate (DEPC)-treated water. Reverse transcription reactions were then performed using 1 μg RNA samples. In animal studies, rats were sacrificed 4 h after provocation, and nasal mucosa was removed from the nasal septum, collected in RNA later, and stored at −80°C prior to assays. Nasal mucosa samples were homogenized using a Polytron (Model PT-K; Kinematica AG, Littau/Luzern, Switzerland) in 10 volumes of ice-cold RNAiso Plus Reagent. Homogenates were then mixed with chloroform and centrifuged at 15,000 rpm for 15 min at 4°C. Aqueous phases containing RNA were then transferred to fresh tubes, and RNA was precipitated by adding isopropanol and centrifuging at 15,000 rpm for 15 min at 4°C. RNA samples were then dissolved in DEPC-treated water, and

reverse-transcription to cDNA was performed using PrimeScript RT reagent Kits. TaqMan primers and probes were designed using Primer Express software (Applied Biosystems; Table 2). Real-time PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems). To standardize the starting material, endogenous control human GAPDH and rodent GAPDH control reagents (Applied Biosystems) were used, and data were expressed as a ratio of GAPDH mRNA.

Primer/probe name	Sequence
Human H1R mRNA	
Sense primer	5′-CAGAGGATCAGATGTTAGGTGATAGC-3′
Anti-sense primer	5′-AGCGGAGCCTCTTCCAAGTAA-3′
Probe	FAM-CTTCTCTCGAACGGACTCAGATACCACC-TAMRA
Rat H1R mRNA	
Sense primer	5′-TATGTGTCCGGGCTGCACT-3′
Anti-sense primer	5′-CGCCATGATAAAACCCAACTG-3′
Probe	FAM-CCGAGAGCGGAAGGCAGCCA-TAMRA
Rat IL-4 mRNA	
Sense primer	5′-CAGGGTGCTTCGCAAATTTTAC-3′
Anti-sense primer	5′-CACCGAGAACCCCAGACTTG-3′
Probe	FAM-CCCACGTGATGTACCTCCGTGCTTG-TAMRA
Rat IL-5 mRNA	
Sense primer	5′-CAGTGGTGAAAGAGACCTTGATACAG-3′
Anti-sense primer	5′-GAAGCCTCATCGTCTCATTGC-3′
Probe	FAM-TGTCACTCACCGAGCTCTGTTGACG-TAMRA
Rat IL-9 mRNA	
Sense primer	5′-GACGACCCATCAAAATGC-3′
Anti-sense primer	5′-CTGTGACATTCCCTCCTGYAA-3′
Probe	FAM-TTGTGCCTCCCCATCCCATCTGAT-TAMRA

A primer probe kit from Applied Biosystems (Rn00587615-A1 1113) was used to determine rat IL-13 mRNA expression.

Table: 4-2. Nucleotide sequences of primers and probes

4.2.8. Immunoblot analysis:

For determination of PKCδ phosphorylation, HeLa cells were serum-starved for 24 h and stimulated with 100 nM of PMA for 10 min in 100-mm dishes. Cells were pretreated with 100 or 250 μM MBCA and 5 μM rottlerin for 1 h before stimulation with PMA. The cells were harvested in TBS containing proteinase inhibitors (Complete Mini, Roch Applied Sciences) and phosphatase inhibitors (Phos STOP, Roch Applied Sciences), and total lysate was prepared by sonication. For determination of H1R expression, HeLa cells were serum-starved for 24 h and stimulated with 100 nM of PMA for 24 h in 100-mm dishes. Cells were pretreated with 100 or 250 μM MBCA and 10 μM rottlerin for 1 h before stimulation with PMA. The cells were harvested with 1.5 ml of ice-cold 50 mM Na_2/K-phosphate buffer, pH 7.4, and membrane fraction was prepared by sonication followed by the centrifugation at 15,000 g x 30 min. For immunoblot analysis, protein samples were separated on 10% SDS-PAGE gels and were transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA, USA). Membranes were briefly rinsed in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and were then incubated for 1 h at room temperature in TBS-T containing 5% skim milk (Difco, BD Japan, Tokyo, Japan) or 3% bovine serum albumin (for detecting phosphoproteins). Membranes were then incubated with primary antibodies against PKCδ (C-20), (sc-937, 1:1000, Santa Cruz Biotechnology), phospho-PKCδ (Tyr^{311}), (#2055S, 1:500, Cell Signaling Technology Japan, Tokyo, Japan), H1R (AB5654P, 1:500, Chemicon International), and β-actin, (#4697, 1:2000, Cell Signaling) overnight at 4°C. Goat anti-rabbit IgG (H + L)-HRP conjugate (#170-6515, 1:10,000, Bio-Rad) or Immun-Star goat anti-mouse-HRP conjugate (#170-5047, 1:10,000, Bio-Rad) was used

as secondary antibodies, and proteins were visualized using Immobilon Western Chemiluminescent HRP substrate (Merck Millipore, Billerica, MA, USA).

4.2.9. Subcellular localization of PKCδ:

To determine subcellular localization of PKCδ, HeLa cells were plated onto 35-mm glass-bottomed dishes (Asahi Techno Glass, Chiba, Japan), and an expression plasmid encoding DsRed-tagged PKCδ (Ds-Red-PKCδ) and pAcGFP-Golgi (for labeling of the Golgi) was co-transfected into attached HeLa cells using PolyFect transfection reagent (Qiagen, Tokyo, Japan) as described previously [62]. After 5 h, the medium was replaced with 1 ml of serum-free medium, and the cells were starved for 24 h. Cells were then stimulated with 100 nM PMA for 20 min in the same medium and were treated with 100 μM MBCA for 1 h before PMA stimulation. After stimulation, cells were washed once in PBS and fixed with ice-cold methanol, and the PBS was then replaced. Subcellular localization of the Ds-Red-PKCδ was determined using a confocal laser microscope (LSM510; Carl Zeiss, Oberkochen, Germany).

4.2.10. Statistical analysis:

Data are presented as means ± standard errors of the mean (S.E.M.). Differences were identified using ANOVA and Dunnett's multiple comparison test with GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA) and were considered significant when $p < 0.05$.

4.3. Results

4.3.1. Effect of MBCA on PMA- or histamine-induced H1R expression in HeLa cells:

Stimulation of HeLa cells with PMA or histamine significantly increased H1R mRNA expression [59]. In contrast, pretreatment with various concentrations of MBCA dose dependently suppressed PMA- and histamine-induced H1R mRNA expression (Fig. 4-3. A and B). Moreover, suppression of H1R mRNA expression by MBCA (50 μM) was comparable with that by the PKCδ-selective inhibitor rottlerin and the conventional antihistamines chlorpheniramine and epinastine (Fig. 4-3.C). MBCA also dose-dependently suppressed PMA-induced up-regulation of H1R protein expression (Fig. 4-3. D).

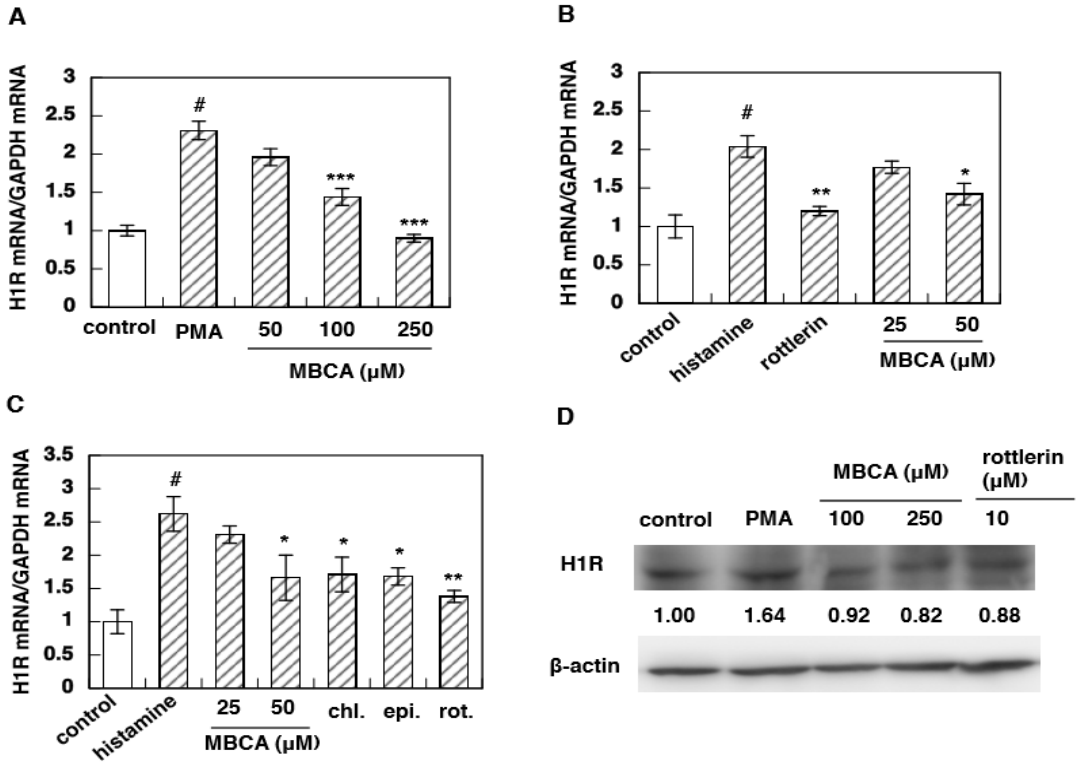


Figure: 4-3. Effects of MBCA on PMA- and histamine-induced upregulation of H1R expression in HeLa cells.

HeLa cells were serum starved for 24 h at 37°C before treatment with 100 nM PMA (A) or 100 μM histamine (B) for 3 h. HeLa cells were preincubated with indicated concentrations of MBCA and 5 μM rottlerin for 1 h before PMA or histamine stimulation. H1R mRNA expression was determined using quantitative reverse transcription-polymerase chain reaction. (C) Comparative activities of antihistamines and rottlerin with MBCA; HeLa cells were preincubated with MBCA (25 and 50 μM), chlorpheriramine (chl., 10 μM), epinastine (epi., 10 μM), and rottlerin (rot., 10 μM) for 1 h before histamine stimulation. In (D), HeLa cells were treated with MBCA 1 h before PMA stimulation. After 24 h stimulation with PMA, membrane fraction was prepared and subjected to the immunoblot analysis to detect H1R protein. Numbers below the blots for H1R indicate relative band intensity normalized to β-actin. Data are expressed as means ± S.E.M.; #$p < 0.0001$ vs. control, ***$p < 0.0001$, **$p < 0.01$, and *$p < 0.05$ vs. PMA- or histamine-treated cells (n = 3–4).

4.3.2. Effect of MBCA on PMA-induced phosphorylation of PKCδ at Tyr^{311}:

Activities, stabilities, and functions of PKCs are regulated by phosphorylation and dephosphorylation reactions, and PKCδ is reportedly activated by phosphorylation at Tyr^{311} [100]. Previously, we demonstrated that stimulation with histamine or PMA led to increased phosphorylation of PKCδ at Tyr^{311} in HeLa cells [62]. Therefore, we investigated the effect of MBCA on PMA-induced phosphorylation of PKCδ at Tyr^{311} and showed that pretreatment with MBCA suppressed PMA-induced phosphorylation (Fig. 4-4.).

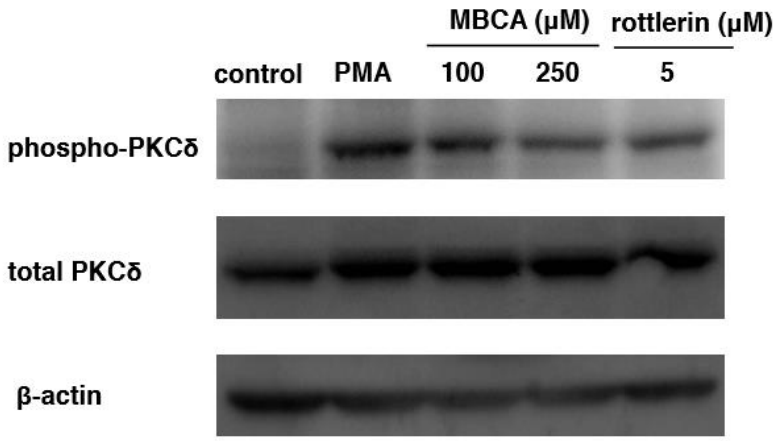


Figure: 4-4. Effect of MBCA on PMA-induced phosphorylation of PKCδ at Tyr^{311}.

HeLa cells were serum starved for 24 h and were then treated with 100 or 250 μM MBCA and 5 μM rottlerin for 1 h before stimulation with 100 nM PMA for 10 min. Total cell lysates were prepared, and phosphorylation of PKCδ at Tyr^{311} was determined using immunoblot analyses.

4.3.3. Effect of MBCA on PMA-induced translocation of PKCδ:

We previously demonstrated that stimulation with histamine or PMA caused translocation of PKCδ from the cytosol to the Golgi, and pretreatment with the PKCδ-selective inhibitor rottlerin inhibited PKCδ translocation [62]. These data indicate that PKCδ translocation to the Golgi is a crucial event for histamine- or PMA-induced upregulation of H1R expression in HeLa cells [62]. Accordingly, pretreatment with MBCA suppressed PMA-induced translocation of PKCδ to the Golgi (Fig. 4-5.).

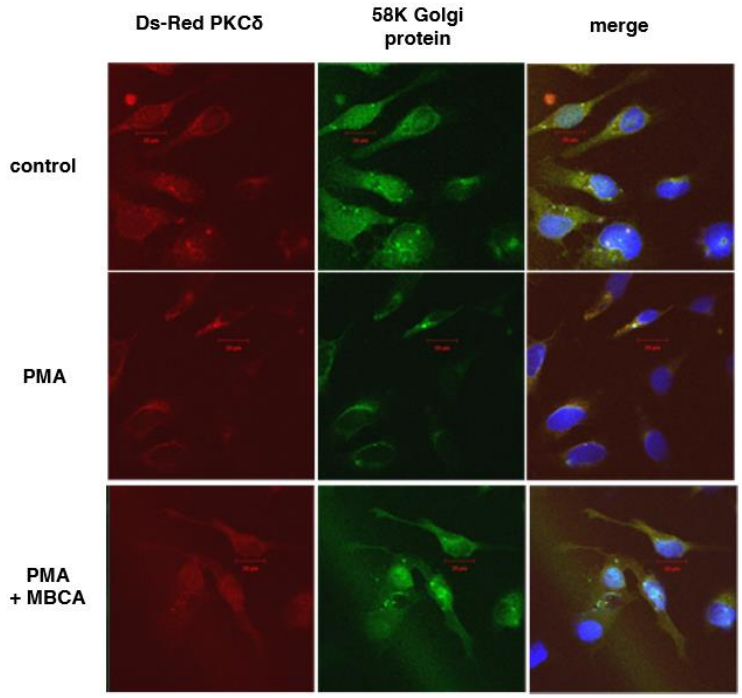


Figure: 4-5. Effect of MBCA on PMA-induced PKCδ translocation.

Expression plasmids encoding Ds-Red-PKCδ and pAcGFP-Golgi were co-transfected into attached HeLa cells on 35-mm glass bottomed dishes as described previously. Cells were serum starved for 24 h and were then stimulated with 100 nM PMA for 20 min in the same medium. Cells were treated with 100 µM MBCA for 1 h before PMA stimulation. Subcellular localization of Ds-Red-PKCδ was determined using a confocal laser microscope. Bar = 20 µm.

4.3.4. Metabolic stability of MBCA on rodent liver microsomes:

Metabolic stability is defined as the susceptibility of a chemical compound to biotransformation and is expressed as *in vitro* half-life ($T_{1/2}$) and intrinsic clearance (CL_{int}) [101]. In accordance with the long half-lives ($T_{1/2}$) of the drugs, we previously

tested once daily drug dosage regimens in TDI-sensitized rats 1 h before intranasal application of TDI [35, 47, 49, 50, 51]. However, we could not see any suppressive effect of MBCA on TDI-induced nasal symptoms and upregulation of H1R gene expression in TDI-sensitized rats using the same regimens (data not present). Thus we conducted *in vitro* kinetic studies of MBCA. In these experiments, MBCA was subjected to oxidative metabolism in rodent (rat and gerbil) and nonrodent (dog, pig, and bovine) liver microsomes. Although MBCA was rapidly metabolized by nonrodent (dog, pig, and bovine) liver microsomes, it showed moderate stability in rodent (rat and gerbil) hepatic microsomes, with $T_{1/2}$ values of 71 and 36 min, respectively (Table 4-3). Furthermore, CL_{int} of MBCA in rodents and nonrodents was calculated from half-lives and were below 40 and above 350 µl/min/mg protein, respectively (Table 4-3). Thus, we designed a twice-daily dosage regimen for animal experiments using TDI-sensitized allergy model rats according to these data.

Microsomes*	Average *T*-half (min)	Average $CL_{int\ app}$ (µl/min/mg protein)
GLM[1]	36.06 ± 0.98	38.45 ± 1.06
RLM[2]	71.65 ± 1.09	19.35 ± 0.30
DLM[3]	3.80 ± 0.071	360.80 ± 5.66
PLM[4]	3.20 ± 0.07	431.90 ± 8.13
BLM[5]	3.00 ± 0.001	462.00 ± 0.00

*liver microsomes from [1]Gerbil, [2]Rat, [3]Dog, [4]Pig, and [5]Bovine.

Table: 4-3. Metabolic stability of MBCA in liver microsomes from various animal species

4.3.5 Effect of MBCA on Caco-2 permeability:

In silico models [102] and high throughput systems, such as parallel artificial membrane permeability assays [103] and cell-based systems [104], are commonly used to assess permeability and oral drug absorption. The most popular high-throughput screening tool for drug permeability utilizes Caco-2 cells [104], because they differentiate upon becoming confluent monolayers and develop tight junctions and polarized apical/mucosal (A side) and basolateral/serosal (B side) membranes that are structurally and functionally similar to those of enterocytes. In the present Caco-2 permeability studies, MBCA was highly labile across apical to basal compartments, with total apparent permeability (P_{app}) of $35.86 \pm 5.55 \times 10^{-6}$ cm/s (Table 4-4). These data indicate that MBCA is transported by passive diffusion through Caco-2 cell monolayers. To determine the roles of P-glycoprotein (P-gp) in Caco-2 permeability, we performed basal to apical permeability studies of MBCA. These experiments demonstrated no affinity of MBCA for P-gp, as reflected by an efflux ratio of about 1, indicating that MBCA is a non-Pgp substrate and permeate Caco-2 cell monolayers by passive diffusion only. Therefore, the present *in vivo* studies were performed after oral administration of MBCA.

Compounds	$P_{app} \times 10^6$ cm/s; $n = 2$			Permeability rank
	A to B (Avg)	B to A (Avg)	Efflux ratio	
MBCA	35.86 ± 5.55	35.93 ± 1.20	1.00 ± 0.03	High
Furosemide	0.09 ± 0.02	15.36 ± 0.23	172.64 ± 2.60	Low
Verapamil	13.81 ± 1.28	75.47 ± 4.30	5.47 ± 0.31	High
Carbamazepine	35.71 ± 2.35	41.78 ± 0.63	1.17 ± 0.02	High

Table: 4-4. Caco-2 permeability of MBCA.

4.3.6. Effect of MBCA on TDI-induced nasal symptoms and increases in H1R and Th2 cytokine mRNA expression in nasal mucosa of TDI-sensitized rats:

We previously identified compounds that suppress H1R upregulation, including (–)-maackiain, epigallocatechin-3′-*O*-gallate, suplatast, and quercetin [35, 39, 50, 51]. These compounds inhibited PMA-induced phosphorylation of PKCδ at Tyr^{311} in HeLa cells and alleviated TDI-induced nasal symptoms and suppressed TDI-induced upregulation of H1R and Th2 cytokine mRNA expression in nasal mucosa of TDI-sensitized rats [35, 39, 50, 51]. Therefore, we investigated the effect of MBCA on TDI-induced nasal symptoms and H1R and Th2 cytokine gene expression in nasal mucosa of TDI-sensitized rats. In these experiments, intranasal application of TDI caused nasal symptoms including sneezing and watery rhinorrhea, and pretreatment with MBCA (15–35 mg/kg) for 3 weeks reduced TDI-induced sneezing, rubbing, and nasal scores (Fig. 4-6) compared with control rats, which showed no nasal symptoms. Repeated application of TDI also increased H1R mRNA expression in the nasal mucosa of TDI-sensitized model rats, with maximal expression after 4 h of TDI provocation [49].

Under these conditions, treatment with MBCA significantly reduced H1R gene expression in nasal mucosa (Fig. 4-7). Th2-cytokines including IL-4, IL-5, IL-9, and IL-13 reportedly play important roles in the allergic inflammation [88]. Repeated application of TDI increased Th2 cytokine expression in nasal mucosa, and whereas MBCA tended to suppress TDI-induced IL-4 mRNA (Fig. 4-8.A), it significantly diminished increases in IL-5, IL-9, and IL-13 mRNA (Fig. 4-8.B–D).

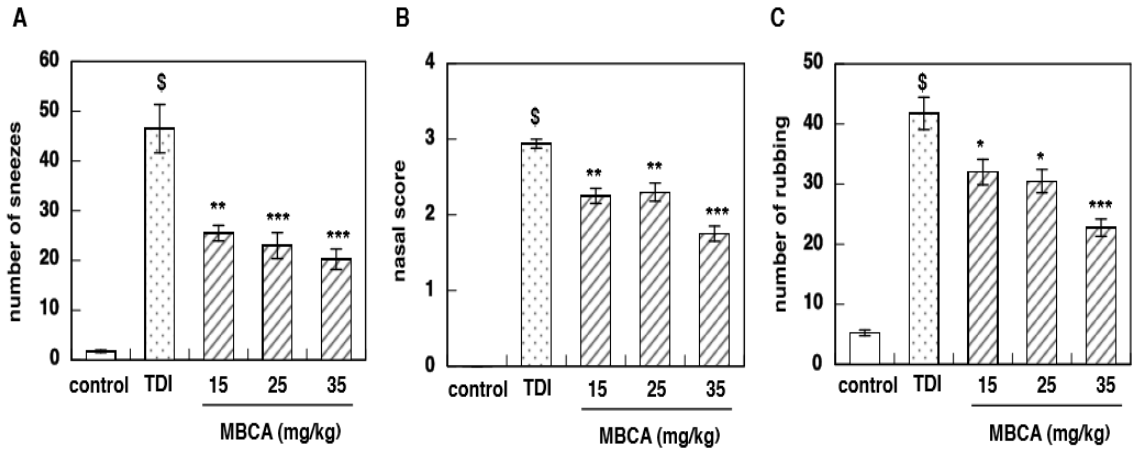


Figure: 8-6. Effect of MBCA on TDI-induced nasal symptoms.

Rats were sensitized and provoked as described in the Materials and Methods section, and numbers of sneezes (**A**) and rubbings (**C**) were counted for 10 min. (**B**) Degrees of swelling, redness, and watery rhinorrhea were scored from 0 to 3 according to the criteria listed in Table 1. Data are expressed as means ± S.E.M.; $^{\$}p < 0.0001$ vs. control, $*p < 0.01$ vs. TDI, $**p < 0.001$ vs. TDI, and $***p < 0.0001$ vs. TDI group (n = 4).

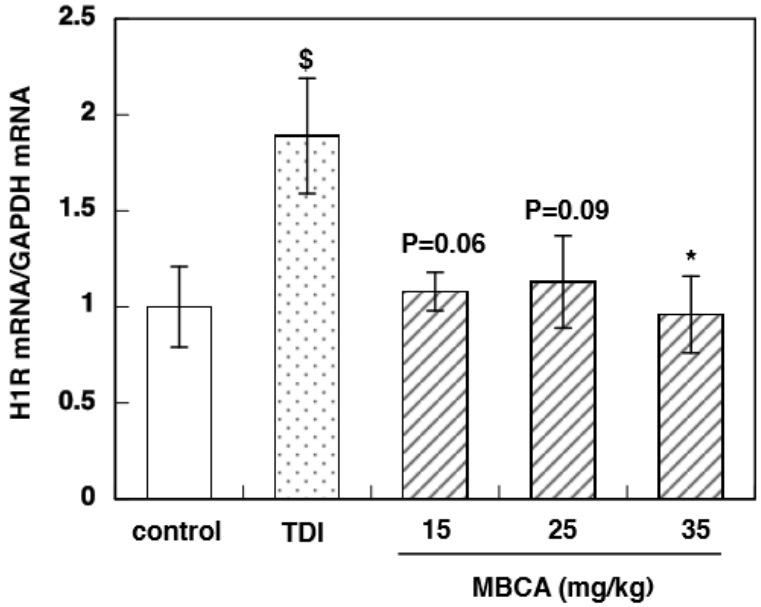


Figure: 4-7. MBCA suppressed TDI-induced upregulation of H1R mRNA expression in nasal mucosa.

Rats were sensitized and provoked as described in the Materials and Methods section and were sacrificed at 4 h, and H1R mRNA expression was determined using reverse transcription-polymerase chain reaction. Data are expressed as means ± S.E.M.; $^{\$}p < 0.05$ vs. control and $^{*}p < 0.05$ vs. TDI ($n = 4$).

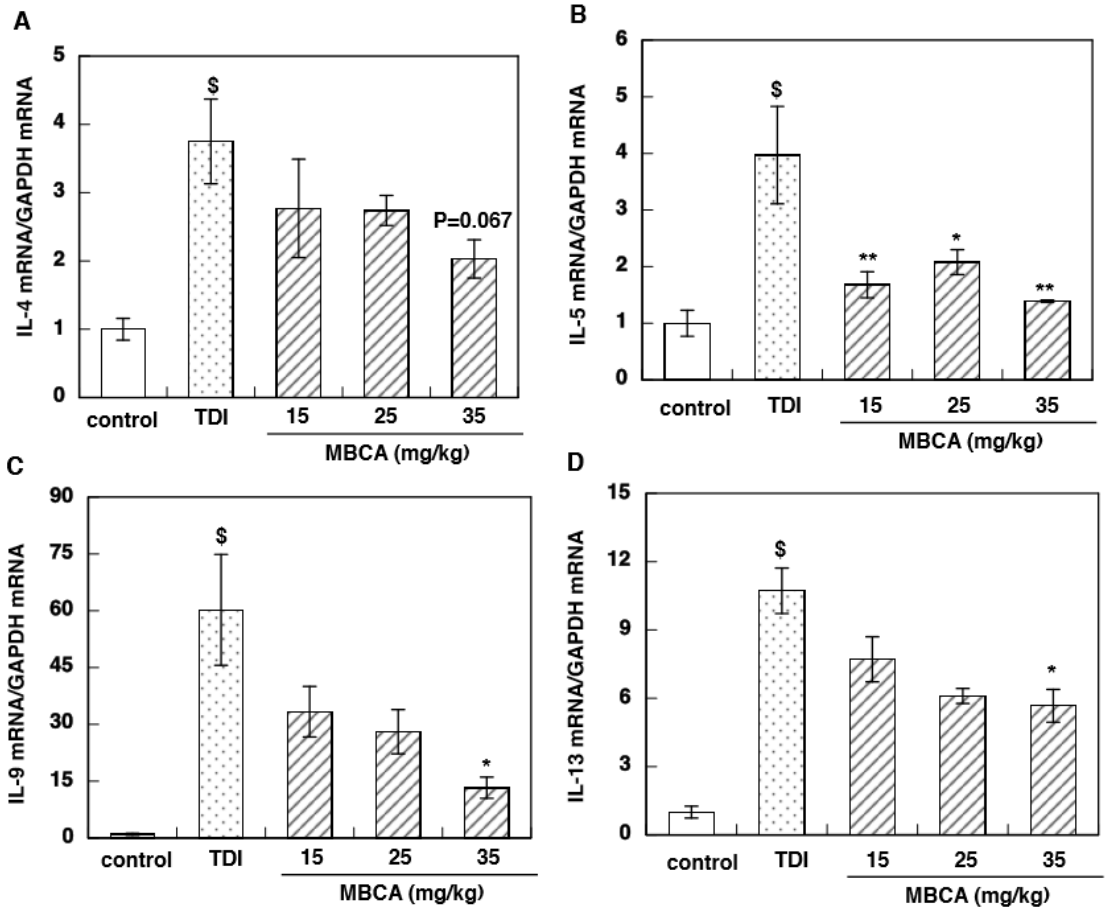


Figure: 4-8. Suppressive effect of MBCA on TDI-induced upregulation of Th2 cytokine gene expression.

Rats were sensitized and provoked as described in the Materials and Methods section and were sacrificed after 4 h, and IL-4 (**A**), IL-5 (**B**), IL-9 (**C**), and IL-13 (**D**). The mRNA expression was determined using reverse transcription-polymerase chain reaction. Data are expressed as means ± S.E.M.; $^{\$}p < 0.001$ vs. control, $^{**}p < 0.01$ vs. TDI, and $^{*}p < 0.05$ vs. TDI ($n = 4$).

4.4. Discussion:

In the present study, we investigated anti-allergic mechanisms of the novel benzofuran MBCA from *T. purpurea*. In a previous study, we demonstrated that H1R gene is an allergic disease-sensitive gene and its expression was correlated with the severity of allergic symptoms [48]. Moreover, compounds that suppressed H1R mRNA expression alleviated TDI-induced nasal symptoms [35, 39, 50, 51], indicating that suppression of H1R signaling may be a promising therapeutic approach for allergic diseases such as AR. We also demonstrated that histamine- and PMA-induced H1R gene expression in HeLa cells was mediated by PKCδ/ERK/PARP-1 signaling, and that binding of histamine to H1R activates PKCδ and leads to its translocation from the cytosol to the Golgi, where PKCδ phosphorylates MEK and ERK.

MBCA suppressed histamine- and PMA-induced H1R mRNA expression in HeLa cells (Fig. 3), warranting the subsequent investigations of MBCA-mediated activation and translocation of PKCδ, which is central to histamine- and PMA-induced H1R expression in HeLa cells. Immunoblot analysis showed that MBCA inhibited phosphorylation of PKCδ at Tyr^{311} (Fig. 4-4). Moreover, pretreatment with MBCA inhibited PMA-mediated PKCδ translocation to the Golgi (Fig. 4-5). Although phosphorylation of PKCδ at Tyr^{311} is not required for membrane recruitment of PKCδ, membrane recruitment was critical for phosphorylation of this molecule at Tyr^{311} [106]. Therefore, inhibition of PKCδ translocation to the Golgi is likely a primary mechanism by which MBCA suppresses upregulation of H1R. The effective dose of MBCA seems high. Ten μM of antihistamines can suppress histamine-induced upregulation of H1R gene expression to the basal level in HeLa cells [59, 107]. Thus, the potency of MCBA suppression is 10-fold weaker than antihistamines. On the other hand, 50 μM of

quercetin was required to suppress PMA-induced upregulation of H1R gene expression to the basal level [51]. In addition, we reported that 30 μM of (–)-maackiain, additional PKCδ signaling inhibitor showed about 50% suppression [39]. Thus, the potencies of these compounds are weaker than antihistamines. Antihistamines bind to H1R with K_B of nM order and inhibit H1R signaling. On the other hand, quercetin and (–)-maackiain suppress H1R signaling by disrupting the interaction of Hsp90 with PKCδ [108]. We think that the difference in IC_{50} of between antihistamines and these compounds are derived from the difference in the mechanism of suppression. We have not checked whether MCBA inhibits the interaction of PKCδ with Hsp90. However, as MBCA suppressed phosphorylation of PKCδ at Tyr^{311} and its translocation to the Golgi, it is likely MBCA also inhibits the interaction of PKCδ with Hsp90.

We also showed upregulation of H1R mRNA and protein in nasal mucosa from TDI-sensitized rats [49, 109], warranting the assumption that increased nasal symptoms in TDI-sensitized rats reflects increased H1R expression in nasal mucosal cells. Accordingly, MBCA significantly suppressed nasal symptoms (Fig. 4-6) and H1R mRNA expression (Fig. 4-7) in TDI-sensitized rats, suggesting that MBCA inhibits nasal hypersensitivity by suppressing TDI-induced H1R expression. However, it is not clear the body concentration of MBCA after its administration. Pharmacokinetic studies showed that MBCA is easily transported by passive diffusion through Caco-2 cell monolayers, but it is rapidly metabolized by microsomes. Thus, it is considered that bioavailability of MBCA is low. Because of its low metabolic stability, the concentration of MBCA in the nasal mucosa may be less than 100 μM after oral administration of MBCA. However, *in vitro* studies, MBCA suppressed PMA-induced

upregulation of H1R gene expression in HeLa cells at the dose of ~100 μM, which is higher than the effective concentration of the in vivo study. One possibility to explain this difference in the effective concentration of MBCA between the *in vivo* and *in vitro* studies is that the nose can accumulate MBCA or that HeLa cells are less sensitive to MBCA than the nasal mucosal cells expressing H1R. However, we have no data to prove these hypotheses. Further studies are required to elucidate the body concentration of MBCA after its administration.

Histamine-cytokine networks, in which histamine influences the expression and actions of several cytokines, have been implicated in several studies showing co-modulation of cytokines and histamine release [22, 23, 110]. Another possibility is that MBCA improve symptoms by the suppression of histamine–cytokine networks, in which MBCA suppresses not only the H1R expression but also Th2 cytokine expression. We showed that antihistamines suppressed TDI-induced upregulation of gene expression of these Th2 cytokines [47]. We also showed that H1R expression is strongly correlated with the gene expression of IL-4 [47], IL-5 [111], IL-9, IL-13, and CD40L (Mizuguchi, Ogishi, and Fukui, unpublished data) in TDI-sensitized rats. In addition, MBCA suppressed TDI-induced upregulation of gene expression of Th2 cytokines (Fig. 4-8). MBCA also suppressed ionomycin-induced upregulation of IL-9 gene expression in RBL-2H3 cells, in which PKCδ signaling pathway is not involved (data not shown). Furthermore, suppression of PKCδ signaling and this unknown signaling that involved in IL-9 gene regulation markedly alleviated nasal symptoms in TDI-sensitized rats (Mizuguchi and Fukui, unpublished data). These findings suggest that gene expression of H1R and Th2 cytokines are correlated and that suppression of H1R gene expression leads to decreased Th2 cytokine production. So, it is possible that

MBCA inhibits both H1R and cytokine production via inhibition of PKCδ and unknown signaling.

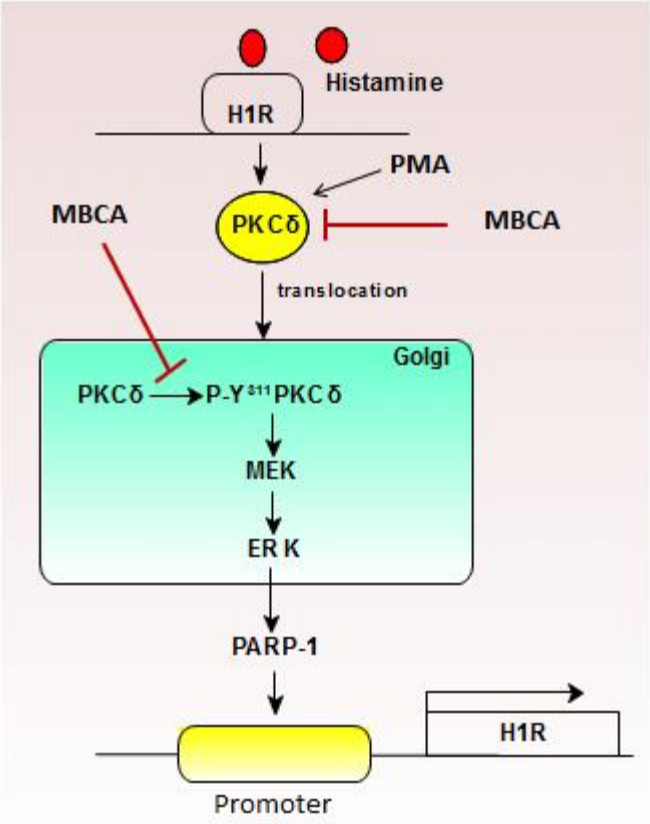


Fig: 4-9. Schematic presentations of the signaling pathway involved in Histamine- and PMA-induced up-regulation of H1R gene expression in HeLa cells and inhibition of PKC-δ translocation and phosphorylation by MBCA.

In summary, MBCA alleviated TDI-induced nasal symptoms and upregulation of H1R and Th2 cytokine gene expression in TDI-sensitized rats. MBCA also inhibited PMA-induced H1R gene expression by suppressing activation of the PKCδ/ERK/PARP-1 signaling pathway. Previously, we characterized the crosstalk

between H1R signaling and Th2 cytokine signaling in TDI-sensitized rats and patients with pollinosis [47, 48, 111]. Therefore, suppression of H1R signaling could inhibit the Th2 cytokine signaling, and H1R signaling is considered to be a crucial target for the treatment of allergies, and the effects of MBCA are likely related to inhibition of TDI-induced upregulation of H1R signaling. The molecular mechanism underlying its suppressive effect for H1R gene expression is mediated by the inhibition of PKCδ activation through the inhibition of PKCδ phosphorylation at Tyr^{311} and blockade of PKCδ translocation to the Golgi.

Section -5

Conclusion

- Medicinal plants have unique attribution to use them as resources for pharmaceuticals, food additives and in fine chemicals.
- *Tephrosia purpurea* (TP) contains abundant of phytochemicals and traditionally used for the treatment of asthma, rheumatism, chronic diarrhea, cirrhosis, and urinary disorders.
- Methanolic extract of TP significantly suppressed PMA-induced up-regulation of H1R mRNA in HeLa cells.
- A novel benzofuran compound, 4-methoxybenzofuran -5-carboxamide (MBCA) was isolated from *Tephrosia purpurea* in a bioassay guided isolation technique.
- Chemical synthesis of MBCA and its derivatives was carried out in a very reproducible synthetic route.
- MBCA suppressed PMA-induced H1R up-regulation at both mRNA and protein level.
- MBCA inhibited PMA-induced phosphorylation of PKCδ at Tyr^{311}.
- MBCA inhibited PMA-induced translocation of PKCδ from the cytosol to the Golgi.
- MBCA suppressed nasal symptoms and H1R mRNA induction in TDI-sensitized rats.
- Mechanism of H1R gene suppression by MBCA underlies the inhibition of PKCδ signaling.

Section -6

Abbreviations

AR: Allergic Rhinitis

MBCA: 4-methoxybenzofuran-5-carboxamide

BSA: bovine serum albumin

ERK: extracellular signal-regulated kinase

FBS: fetal bovine serum

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

H1R: histamine H_1 receptor

IL: interleukin; PKCδ: protein kinase C-δ

PMA: phorbol 12-myristate 13-acetate

RT-PCR: reverse transcription-polymerase chain reaction

TDI: toluene-2,4-diisocyanate

MEM: minimum essential medium

HPLC: high performance liquid chromatography

Th2: helper T cell type

NMR: Nuclear Magnetic Resonance Spectroscopy

HRMS: High Resolution Mass Spectroscopy

IR: Infra-Red Spectroscopy

Section -7

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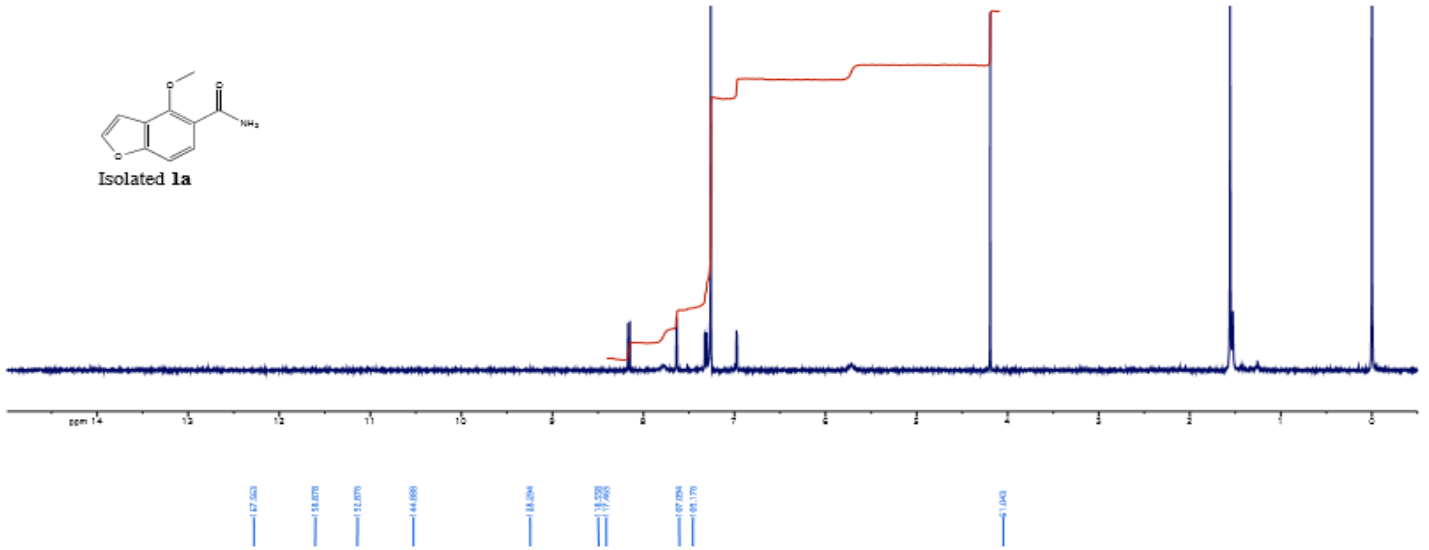
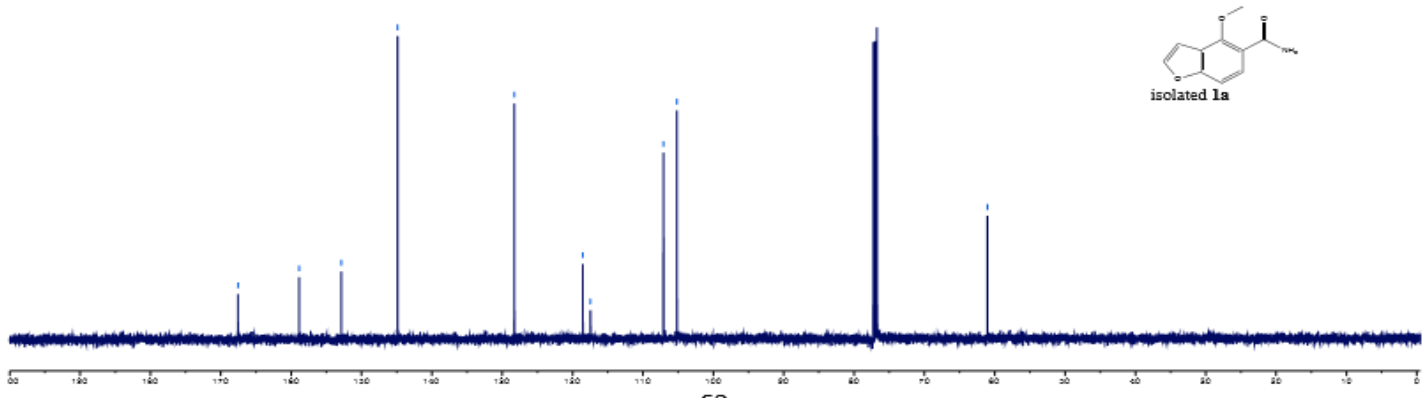
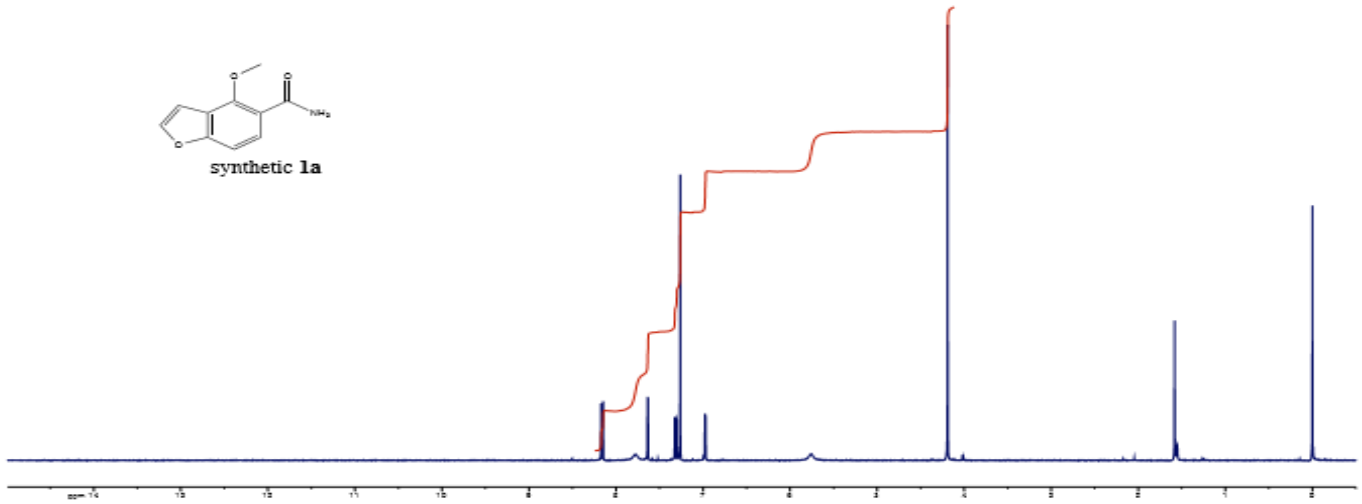
Appendix

^{1}H NMR and ^{13}C NMR of Compounds (S2–S12)

2D-NMR of isolated 1a


Isolated 1a


isolated 1a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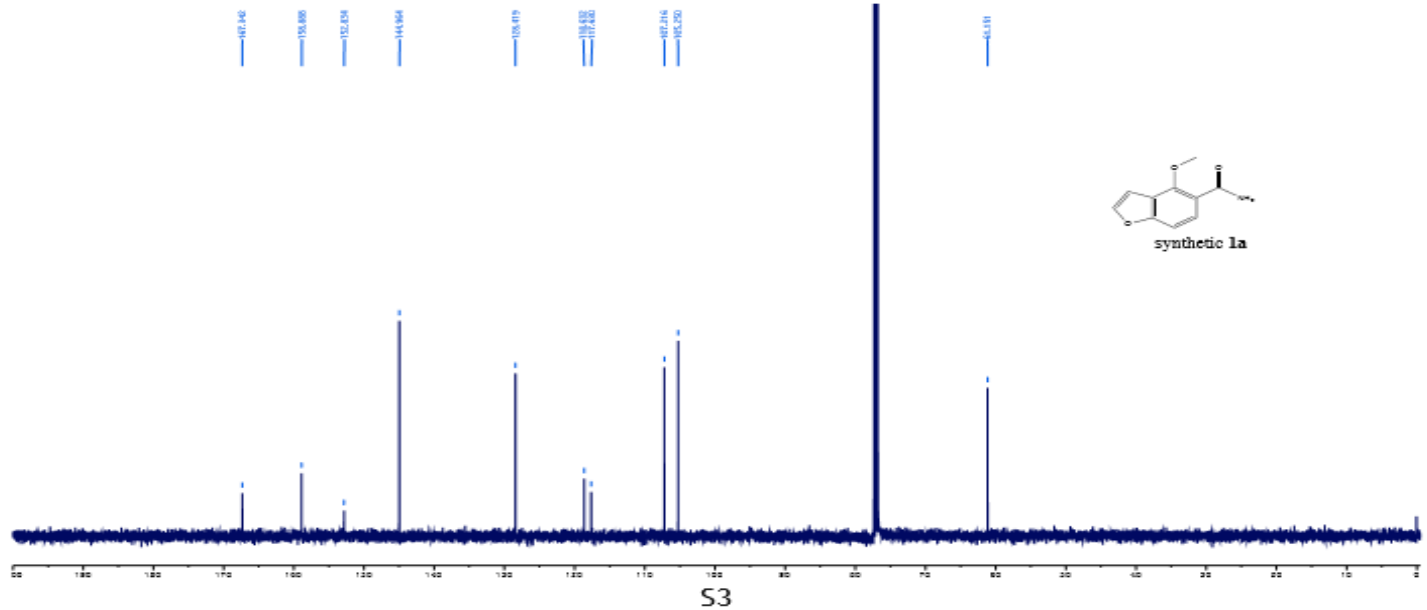
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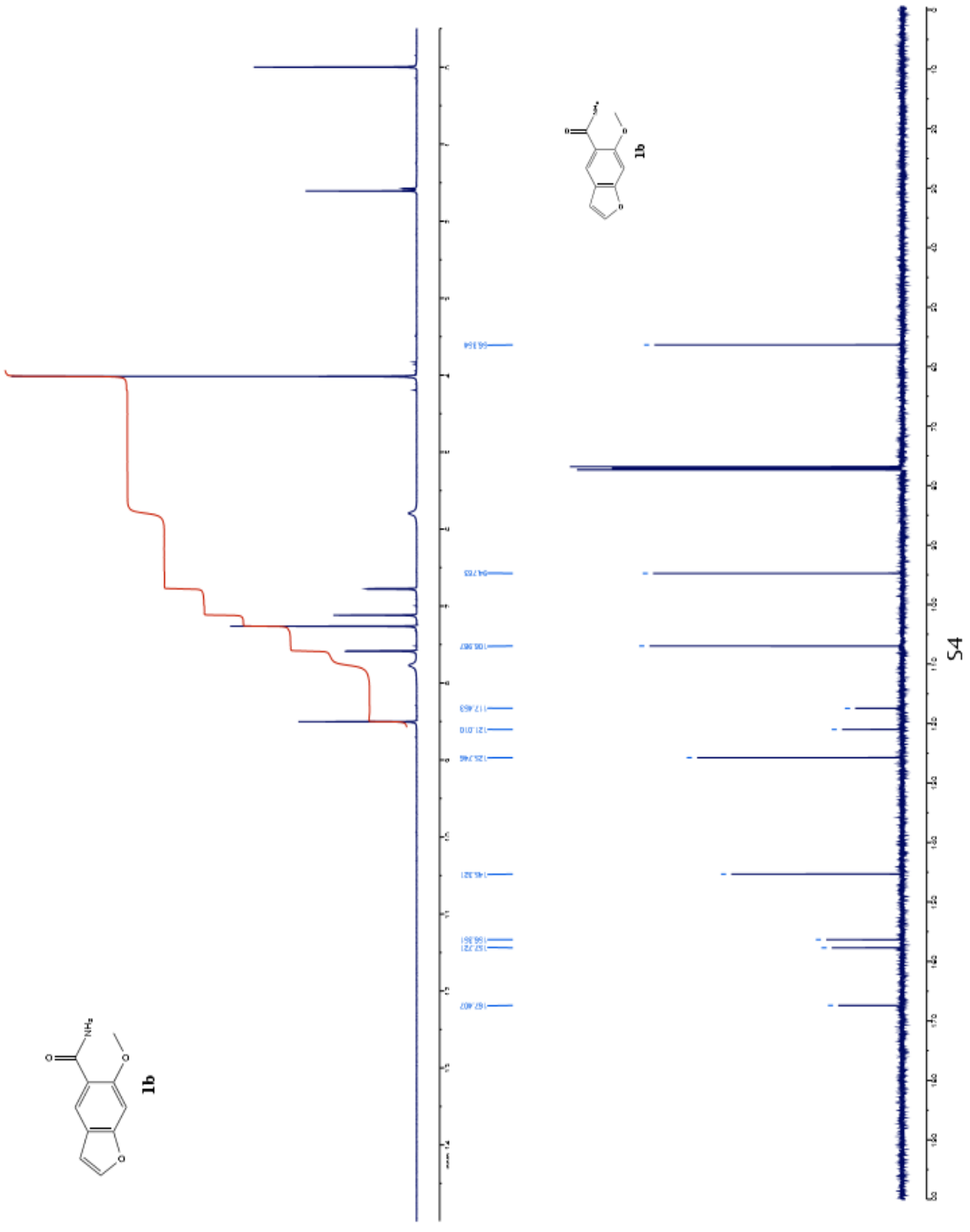
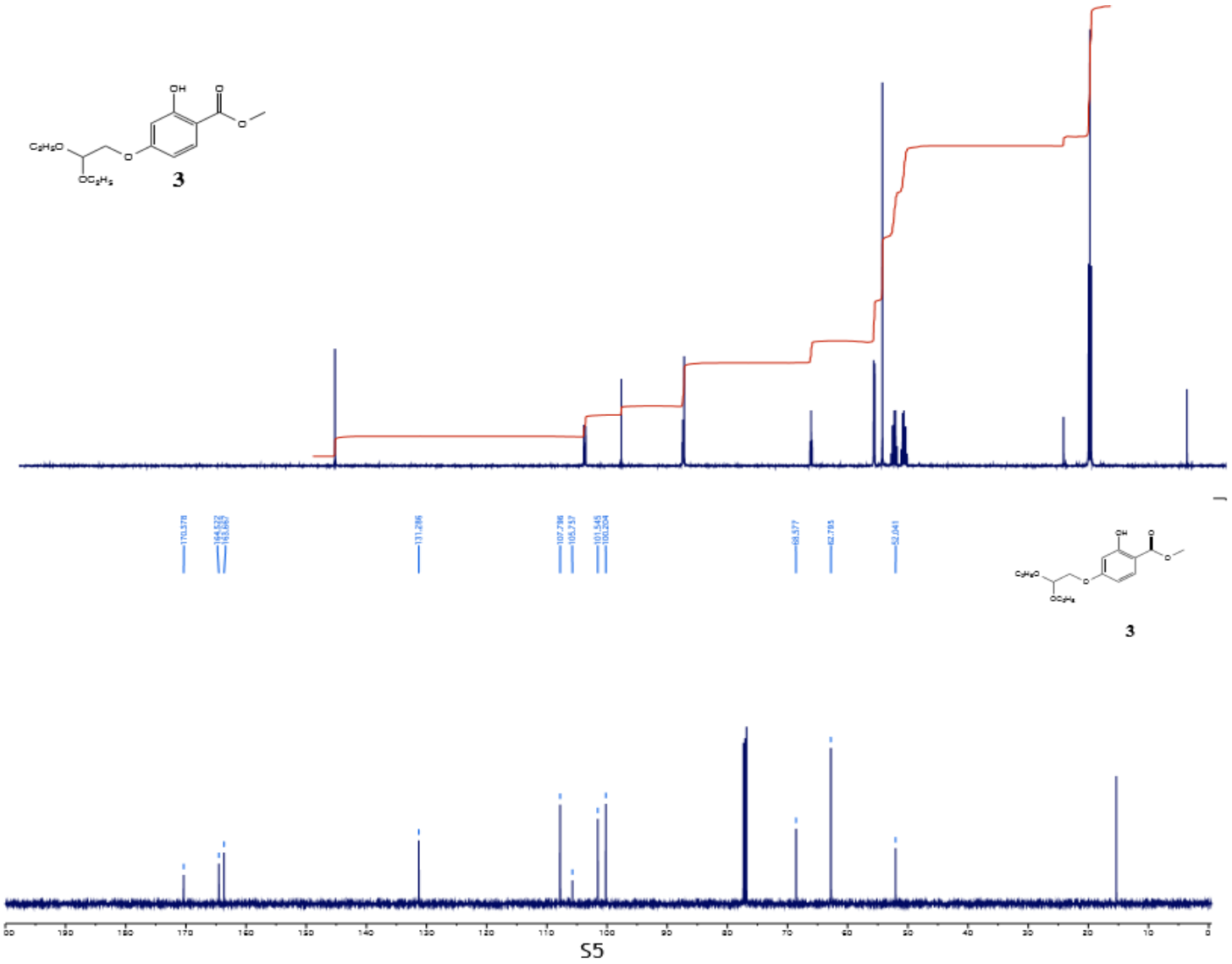

1b
1b
S4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O
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131.286
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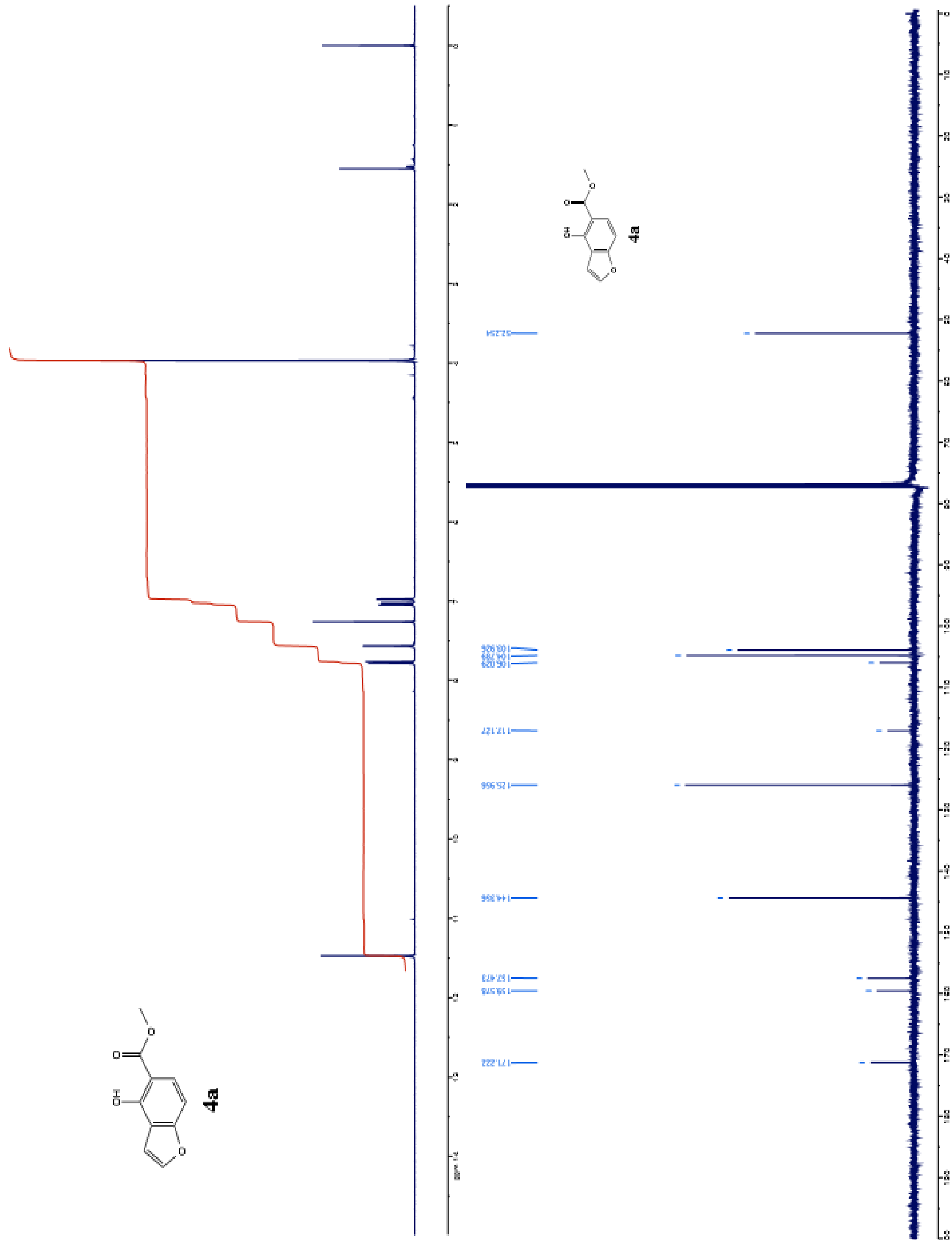
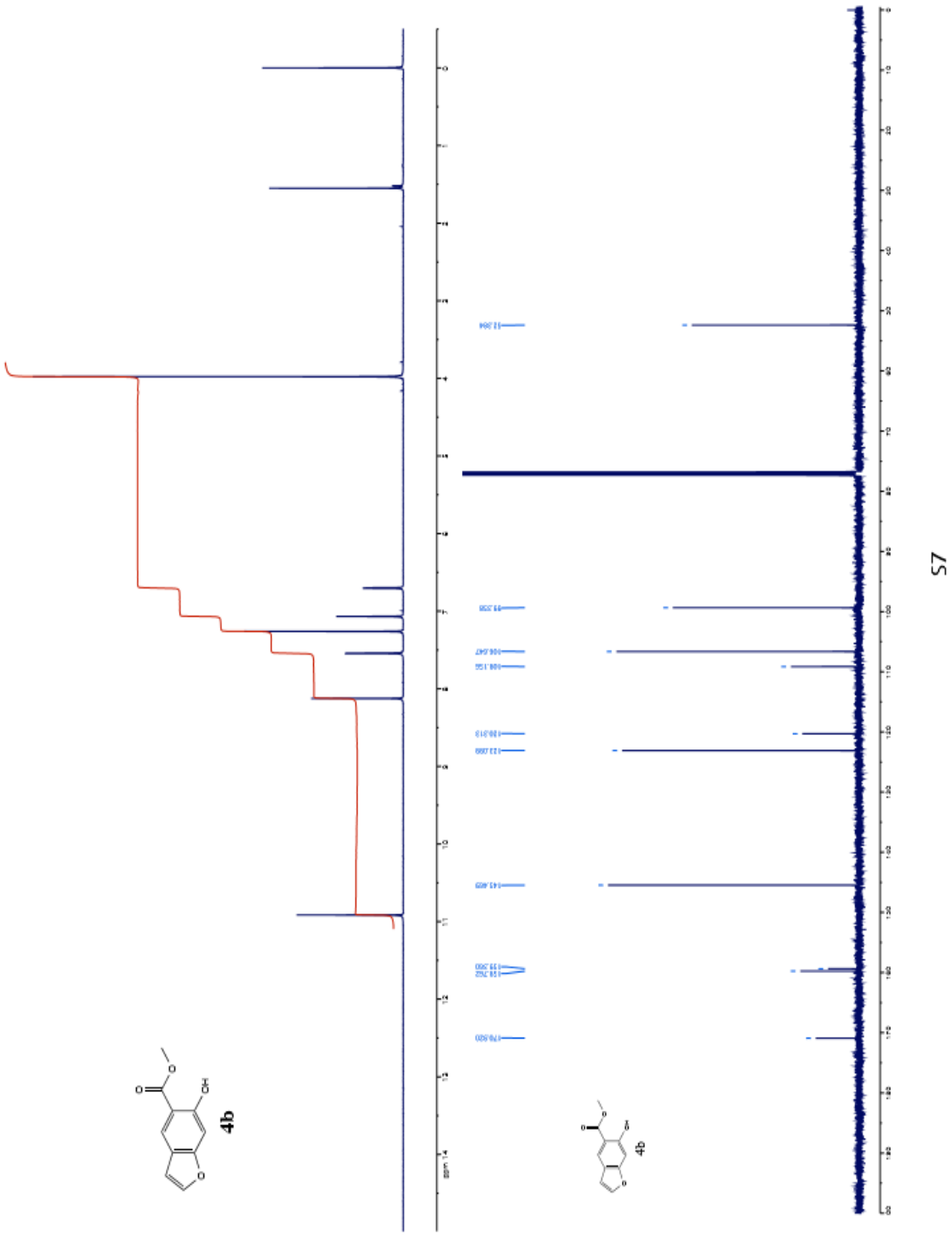
101.545
100.204
68.377
62.765
52.041
3



S6



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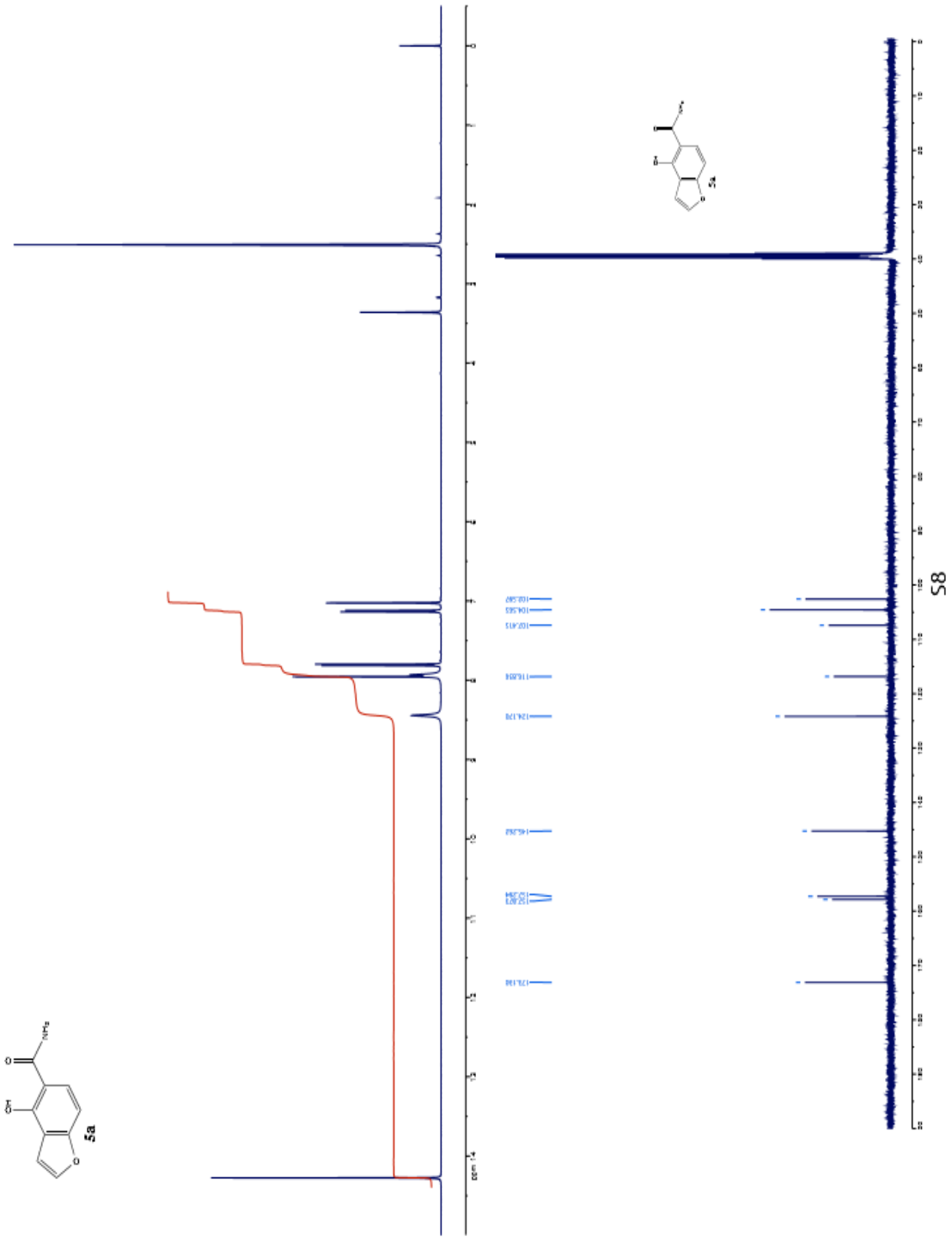
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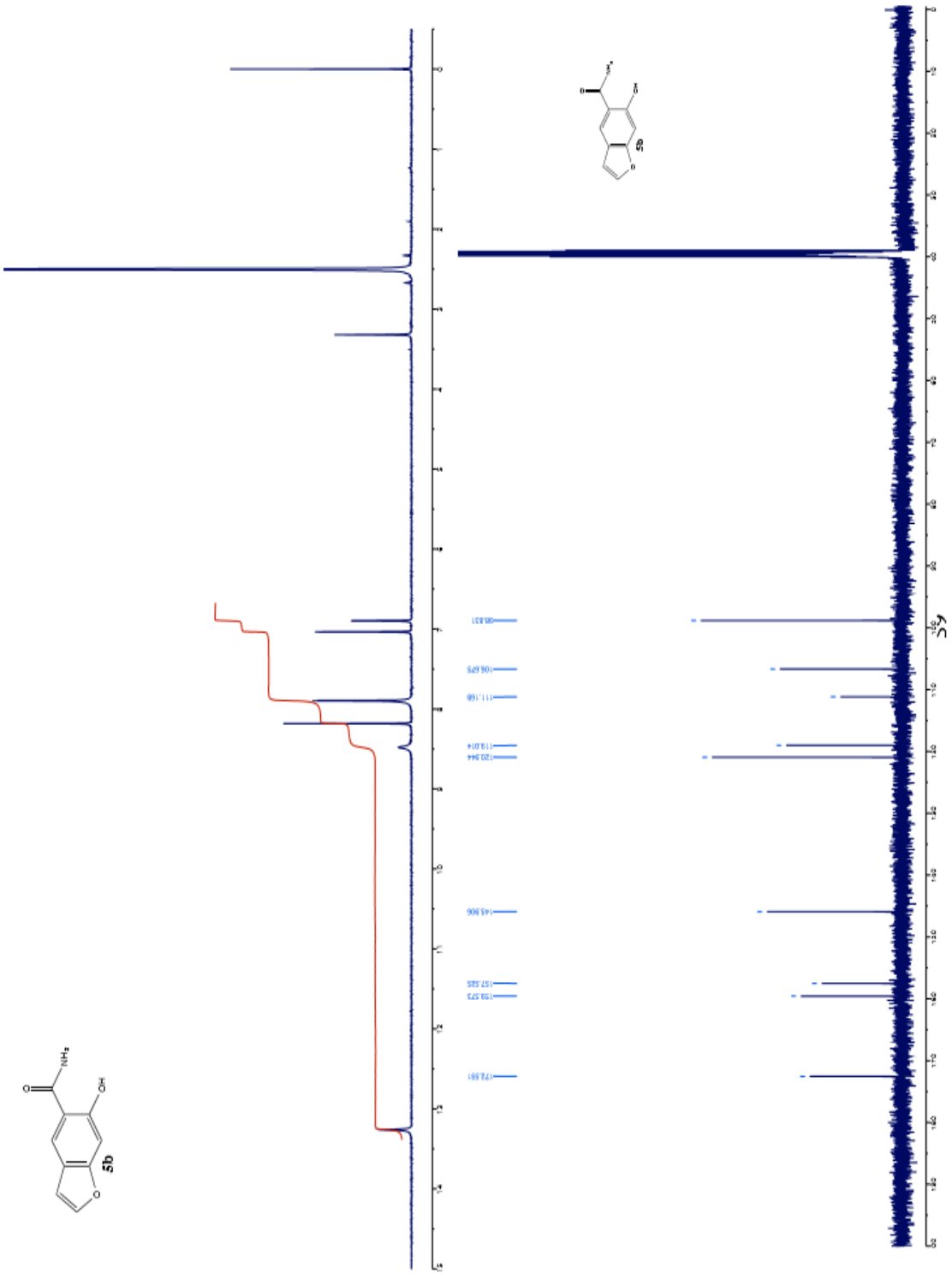
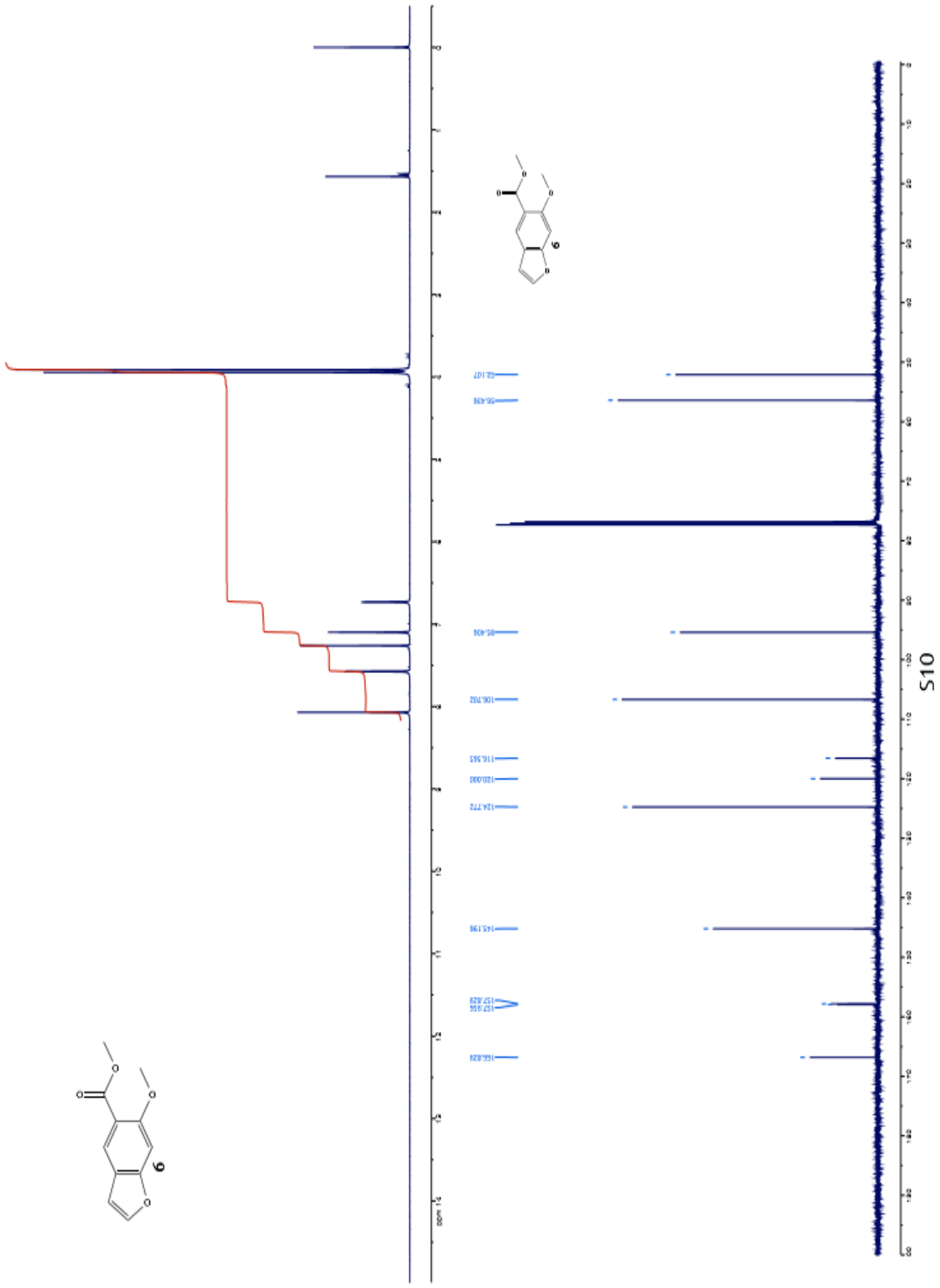
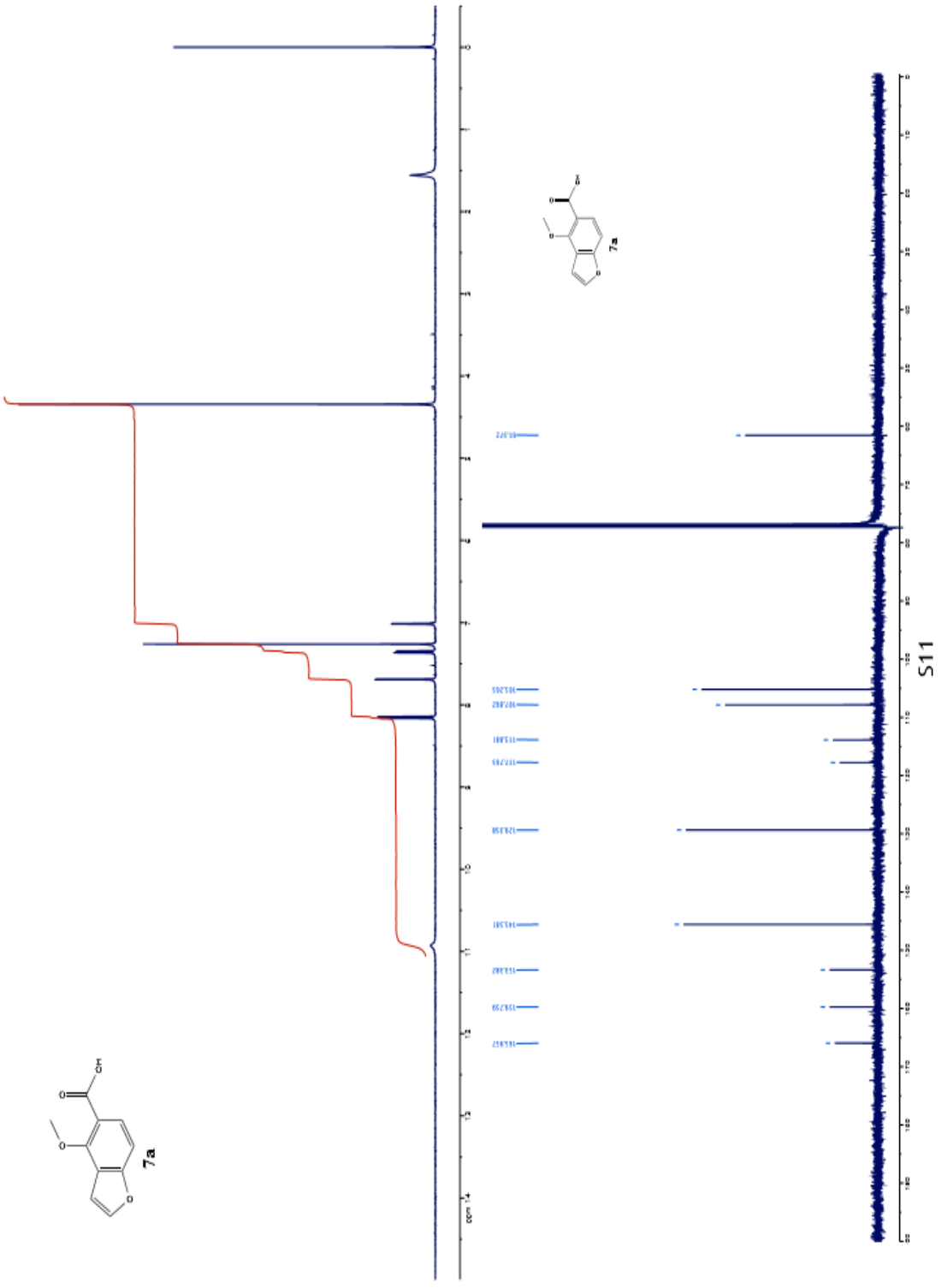
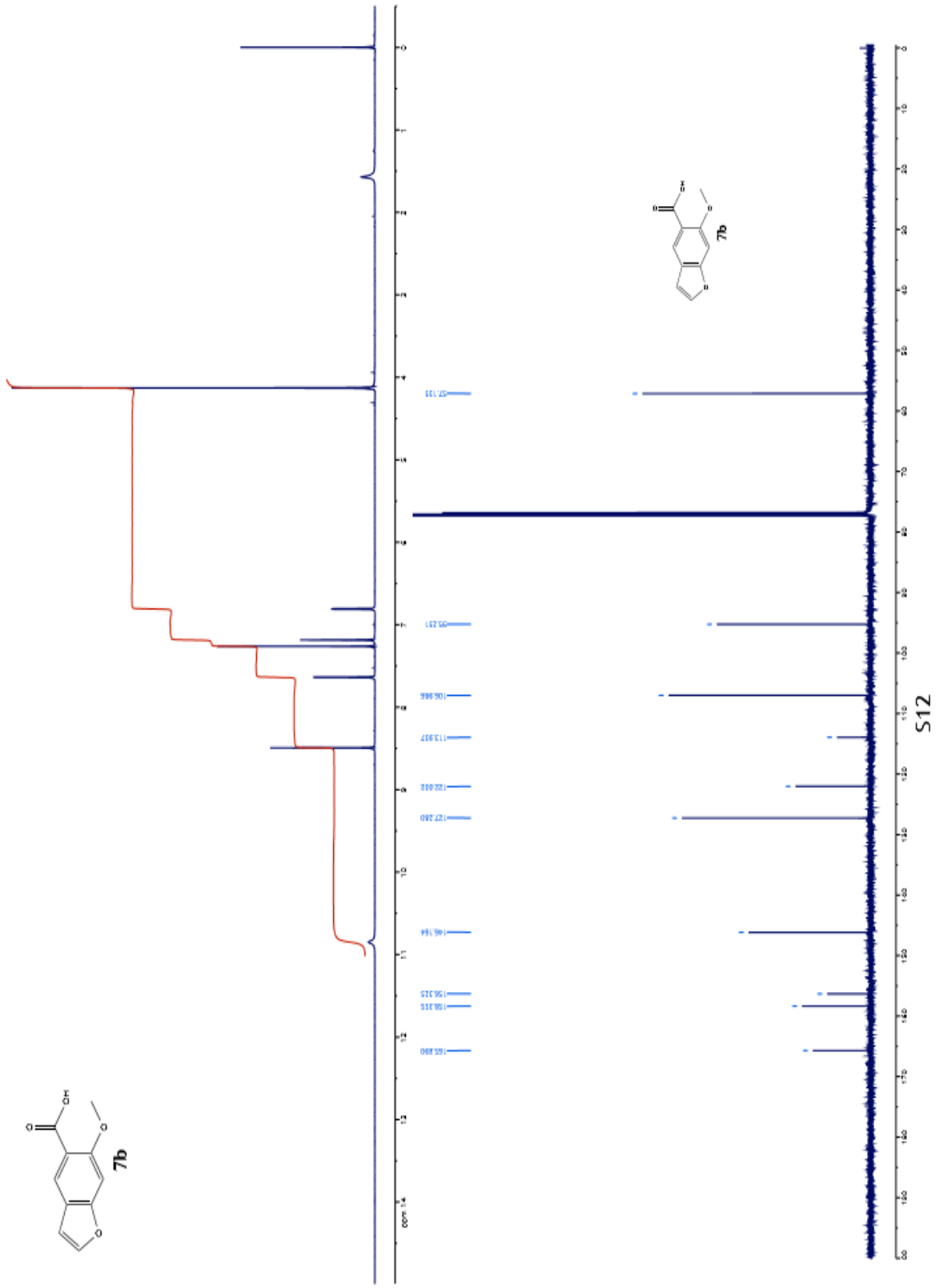
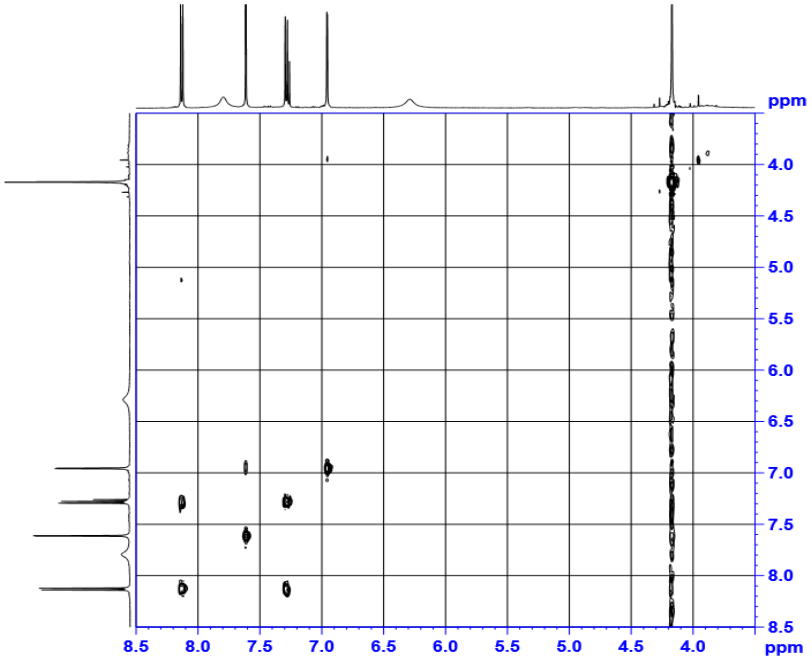
5s




9
9
S10


7a
7a
S11


7b
7b
S12

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TD                      2048
SOLVENT                 CDCl3
NS                         4
DS                         8
SWH                 5000.000 Hz
FIDRES              2.441406 Hz
AQ                  0.2048500 sec
RG                        203
DW                  100.000 usec
DE                    6.50 usec
TE                    302.3 K
D0                  0.00000300 sec
D1                  1.45576203 sec
D13                 0.00000400 sec
D16                 0.00010000 sec
IN0                 0.00019995 sec

======== CHANNEL f1 ========
NUC1                       1H
P0                       9.70 usec
P1                       9.70 usec
PL1                      1.00 dB
PL1W            19.45280783 W
SFO1            500.1425007 MHz

====== GRADIENT CHANNEL =====
GPNAM1                SINE.100
GPZ1                     10.00 %
P16                    1000.00 usec

F1 - Acquisition parameters
TD                         128
SFO1                500.1425 MHz
FIDRES              39.079631 Hz
SW                     10.000 ppm
FnMODE                      QF

F2 - Processing parameters
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SF                  500.1400166 MHz
WDW                       SINE
SSB                  0
LB                   0 Hz
GB                   0
PC                        1.40

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WDW
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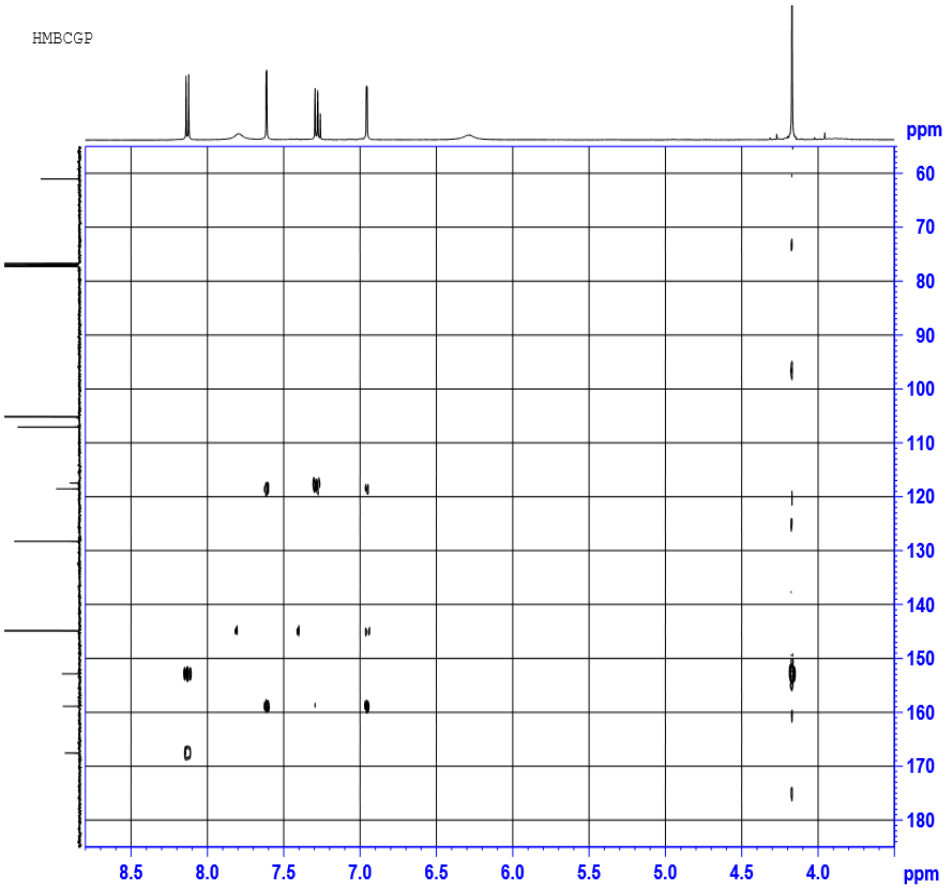


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PROCNO                       1

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PULPROG          hmbcgplpndqf
TD                      4096
SOLVENT                 CDCl3
NS                        16
DS                        16
SWH                 5000.000 Hz
FIDRES              1.220703 Hz
AQ                  0.4098500 sec
RG                        203
DW                  100.000 usec
DE                    6.50 usec
TE                    301.5 K
CNST2               145.0000000
CNST13               7.0000000
D0                  0.00000300 sec
D1                  1.44592298 sec
D2                  0.00344828 sec
D5                  0.07142857 sec
D16                 0.00010000 sec
IN0                 0.00001990 sec

======== CHANNEL f1 ========
NUC1                       1H
P1                       9.70 usec
P2                      19.40 usec
PL1                      1.00 dB
PL1W            19.45280783 W
SFO1            500.1425007 MHz

======== CHANNEL f2 ========
NUC2                      13C
P3                      11.40 usec
PL2                  0 dB
PL2W            100.97783661 W
SFO2            125.7728790 MHz

====== GRADIENT CHANNEL =====
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GPNAM2                SINE.100
GPNAM3                SINE.100
GPZ1                     50.00 %
GPZ2                     30.00 %
GPZ3                     40.10 %
P16                    1000.00 usec

F1 - Acquisition parameters
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SFO1                125.7729 MHz
FIDRES              299.459228 Hz
SW                    200.000 ppm
FnMODE                      QF

F2 - Processing parameters
SI                        2048
SF                  500.1400186 MHz
WDW                       SINE
SSB                  0
LB                   0 Hz
GB                   0
PC                        1.40

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WDW
SSB                  0
LB                   0 Hz
GB                   0
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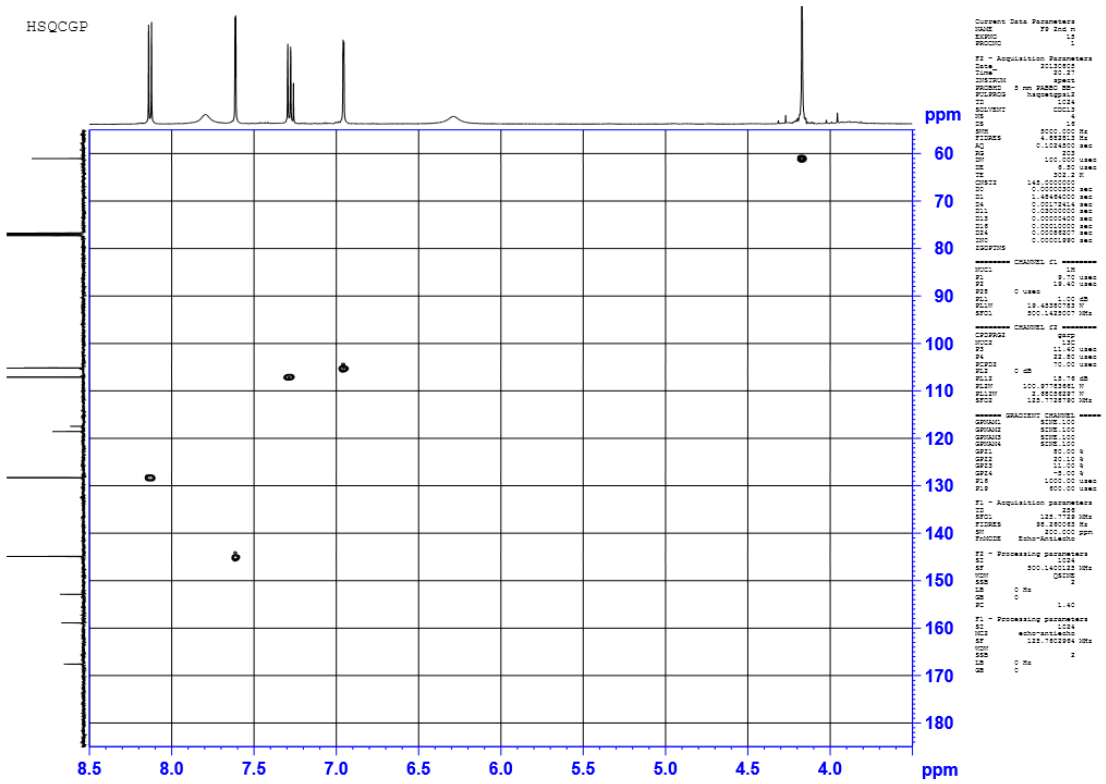
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4.0
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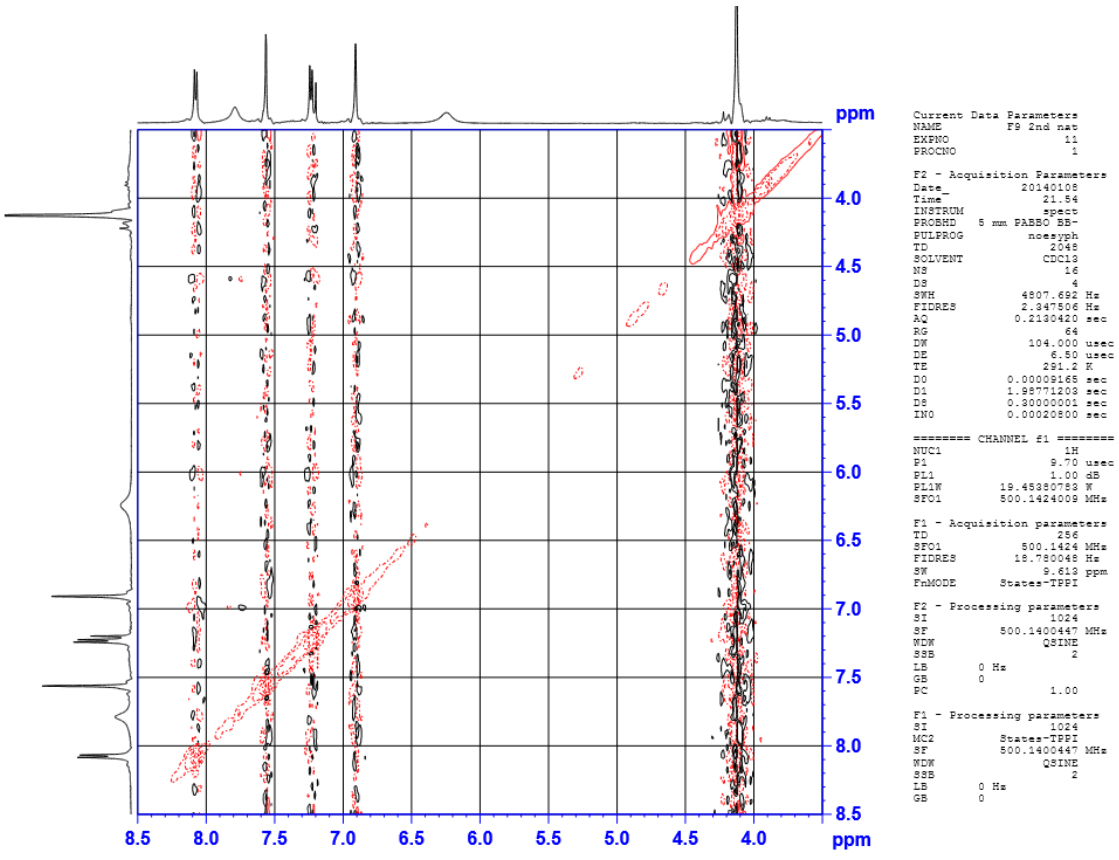
70
80
90
100
110
120
130
140
150
160
170
180

S15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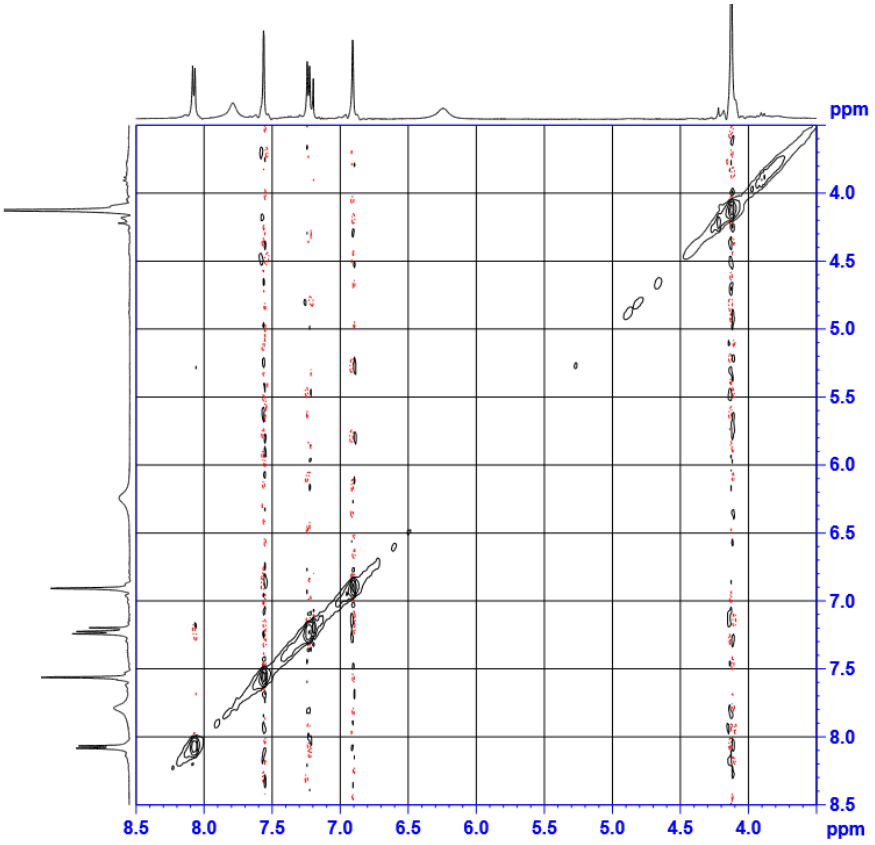
5.5
5.0
4.5
4.0

S16

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PROCNO                 1

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PULPROG          roesyph
TD                  2048
SOLVENT             CDCl3
NS                    16
DS                     4
SWH              4807.692 Hz
FIDRES           2.347506 Hz
AQ               0.2130420 sec
RG                    64
DW                104.000 usec
DE                  6.50 usec
TE                291.2 K
D0              0.00009282 sec
D1              1.95771203 sec
D12             0.00002000 sec
IN0             0.00020800 sec

======== CHANNEL f1 ========
NUC1                  1H
P1                   9.70 usec
P15             200000.00 usec
PL1                  1.00 dB
PL11                21.26 dB
PL1W          19.45280762 W
PL11W          0.18323338 W
SFO1          500.1424009 MHz

F1 - Acquisition parameters
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SFO1           500.1424 MHz
FIDRES          18.780048 Hz
SW                  9.613 ppm
FnMODE       States-TPPI

F2 - Processing parameters
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SF           500.1400447 MHz
WDW                 QSINE
SSB                     2
LB          0 Hz
GB          0
PC                  1.00

F1 - Processing parameters
SI                  1024
MC2          States-TPPI
SF           500.1400447 MHz
WDW                 SINE
SSB                     2
LB          0 Hz
GB          0
```