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RESEARCH ARTICLE

Whole-cell Lipase Catalytic Synthesis of Short-chain Fragrance Esters Using *Aspergillus flavus*

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Abstract: ***Background*:** Fragrances are the collection of unlike functional assemblies, most likely alcohols, esters, aldehydes, ketones, and acids in organic products/hydrocarbons. Short-chain aliphatic fragrance esters have immense applications as flavors in the food, pharmaceutical and cosmetic industries and also have remarkable commercial significance in cosmetics and personal care products like perfumes, face creams, shampoos, soaps, lotions, jams, jellies, *etc*.

***Objective*:** This study aimed to synthesize short-chain fragrance esters using a whole-cell lipase catalyst from Aspergillus flavus (RBD-01).

***Methods*:** The present study emphasizes the synthesis of artificial flavoring compounds by using a whole-cell biocatalytic process, which can have wide significance. Herein, the preparation of ethyl alkanoates (ethyl propanoate to ethyl decanoate) was performed to investigate the flavors and fragrance excellence. The biomass from Aspergillus flavus (RBD-01) was used as a catalyst to facilitate the remarkable esterification activities towards the synthesis of important aroma esters with the help of a series of short-chain acids and alcohols.

***Results*:** The ethyl hexanoate (4) among all synthesized alkanoates was found to have a fruity fragrance with a good conversion rate. Further synthesized alkyl hexanoates (4A-4I) were found to have good fruity/pineapple/berry flavors and significant aroma quality.

***Conclusion*:** These results implied that whole-cell lipase of Aspergillus flavus (RBD-01) is a promising biocatalyst in the production of flavor aroma esters and can boost production in the food/cosmetic manufacturing industries.

ARTICLE HISTORY

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1. INTRODUCTION

Fragrances are a collection of functional assemblies, most likely alcohols, esters, aldehydes, ketones, and acids in organic products/hydrocarbons [1, 2]. The less volatility and low molecular weight (< 400Da) of organic molecules have been found to be significantly effective due to their fragrance in today's generation [2]. Flavor esters are short-chain volatile fragrant compounds with pleasant fruity aromas and remarkable commercial significance in food, beverages, pharmaceuticals, cosmetics, and personal care products like perfumes, face creams, shampoos, soaps, lotions, jams, jellies, *etc*. [1, 3]. Since the reports on the production of fragrance esters from natural plant and animal sources are reasonably limited due to resource constraints and biodiversity concerns, there is significantly more inclination of researchers to explore the synthesis of such compounds through the biocatalytic approach.

Biocatalysis is a specific function of pure enzymes or whole cells (bacteria, algae, fungi, plants, *etc*.) for organic compounds' chemical transformation, facilitating regio/chemo and stereo-selective specificity [4, 5]. This process has gained prominence as a green and sustainable alternative over chemical catalysis due to better substrate selectivity, milder reaction conditions, minimal steps for reaction, less energy utilization, and the formation of significantly less/no harmful by-products [4, 6]. Whole-cell biocatalysts are much preferred over purified enzymes due to their cost-effectiveness, ease of production, and the facilitation of a much greener

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approach [6, 7]. Additionally, whole-cell biocatalysts facilitate economically feasible catalytic processes where the enzyme isolation and purification steps can be easily omitted with much better efficiency in multistep reactions [8, 9]. The use of intracellular lipases in whole-cell biocatalysts is now widely exploited in food, beverages, agricultural, and cosmetics fields [10]. Though plants and animals are considered good sources of lipase production, high yields of lipases have been obtained from different microbial sources, which are widely applied in organic synthesis [11]. Among the established whole-cell biocatalyst systems, filamentous fungi have proven to be robust organisms for industrial applications.

Our earlier study demonstrated the importance of the whole cell catalyzed transesterification using *Aspergillus flavus* (RBD-01) to generate biodiesel from acid oil containing 55 % free fatty acids by conversion of alkyl esters to the extent of 98%. The results revealed that bio catalyzed transesterification process is a unique way to utilize acid oil as feedstock for the production of fuel-grade biodiesel [12]. In the present study, we further demonstrate the use of this fungal whole-cell catalyst for the generation of fragrance esters from aliphatic acid by esterification. Enzymatic expression of *A.flavus* as a whole-cell biocatalyst seems to exhibit a higher catalytic effect when compared with studies carried out by Garlapati *et al*. [13], leading to the synthesis of fragrance esters with a high yield of 94% for nonanol and 84% in case of ethyl decanoate.

2. MATERIALS AND METHODS

2.1. Chemistry Methods

To carry out the experimental work, the laboratory scale culture media and other media constituents *viz.,* mycological peptone, Bushnell Haas broth (BHB), potato dextrose broth (PDB), and potato dextrose agar (PDA) were purchased from HiMedia laboratories, India. Other chemicals, such as aliphatic alcohols (methanol to decanol), aliphatic acids (propionic to decanoic), hexane, ethyl acetate, silica gel *100-200 mesh* for column chromatography, Bi-ammonium hydrogen ortho-phosphate ($(NH_4)_2HPO_4$), were procured from SD Fine-Chem Limited, India. All reagents used were of analytical grade. The solvents used in the compounds' isolation were distilled before use to carry out the present work. The purified compounds were monitored by TLC on 0.25 mm silica gel 60 F254 plates (Merck) and visualized with 2% ceric ammonium sulfate solution as spraying reagents for detecting spots on the TLC under UV illumination. Confirmation of the compounds was initially done by column chromatography using silica gel 100-200 mesh stationary phase. ^{1}H NMR and ^{13}C NMR spectra were recorded on a 400 MHz NMR spectrometer (400 MHz; JEOL JNM-ECS 400) with CD_3Cl_3 as solvent with TMS as an internal standard. The chemical shifts were represented in δ ppm and coupling constants in Hertz. The abbreviations used were as follows: s, singlet; bs, broad singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet. The spectral data were consistent with the assigned structures. A high-resolution mass spectrum (HRMS) and MS were recorded on an Agilent system (Technologies 6540) instrument.

2.2. Preparation of Biomass for use as a Whole-cell Biocatalyst

The 2 to 3 discs (5 mm) were cut from freshly grown culture on PDA plates of *A. flavus* (RBD-01) and inoculated aseptically in 500 ml Erlenmeyer flasks containing 200 ml of sterile PDB. The fully grown fresh culture was then inoculated aseptically into 200 ml minimal media containing nitrogen (mycological peptone (0.5% w/v), (NH4)2HPO4 (0.5%w/v), and carbon source (cottonseed oil (10% v/v). The inoculated flasks were incubated at 28 ± 2°C on an orbital shaker set at 120 rpm. The culture was grown for about 120 h. Fungal biomass was separated from the culture broth by filtering through Whatman filter (No.2) paper, washed with hexane to remove the excess oil, and dried on absorbing paper. The partially dried biomass was crushed vigorously in liquid nitrogen to make a homogenous powder using a sterile pestle mortar.

2.3. Optimization of Esterification Reaction Conditions

Propionic acid was taken as a model aliphatic acid to optimize reaction conditions for the esterification. Furthermore, various aliphatic acids were taken to carry out stepwise modifications to achieve the optimum yield of alkyl esters (Table **1**).

2.4. Identification, Purification, and Quantification of Fragrance Ester

The product (aliphatic ester), thus obtained, was examined on thin layer chromatography (TLC) with hexane: ethyl acetate (98:2) as a mobile phase. The compound was then purified by column chromatography (99:1) and further confirmed on TLC plates by charring with anisaldehyde and potassium permanganate. All reactions were carried out in triplicate, and the obtained alkyl ester products were quantified using ^{1}H-NMR (JEOL 400 MHz,^{1}H-NMR) with $CDCl_3$ as solvent. The chemical shifts were expressed in parts per million with tetramethyl silane (TMS) as the internal standard. ^{1}H NMR spectra were recorded with a pulse duration of 2.18 sec., with a relaxation delay of 4 sec, and 16 scans. All esterification products were quantified by NMR spectroscopy using the (Eq. **1**) proposed by Sharma *et al*. [14]:

$$C = 100 \times (AE\alpha\text{-}\ CH_2 / A\alpha\text{-}\ CH_2) \quad (1)$$

Wherein:

C: conversion of acid to the corresponding alkyl ester.

AEα- CH_2: integration value of the protons of the alkyl esters.

Aα-CH_2: integration value of the methylene protons

3. RESULTS AND DISCUSSION

3.1. Morphology Study of Biomass

As reported earlier, the dry biomass of the whole cell biocatalyst and the filtrate collected from the culture broth were taken up for analysis of lipolytic activity [14]. The culture had a characteristic morphology showing conidiophores of variable length, which were rough and spiny, and the bi-

Table 1. Optimization of parameters for esterification reaction using dried biomass.

S. No.	Constant Parameter	Variable Parameter	Range
Step 1	The molar ratio of propionic acid/ethyl alcohol 1:1 + addition of alcohol at 0 h and 36 h reaction time	Amount of biomass	500 mg to 2000 mg
Step 2	1000 mg biomass + addition of alcohol at 0 h and 36 h reaction time	The molar ratio of ethanol	0.5 to 2.0
Step 3	1000 mg biomass + molar ratio of acid/alcohol 1:1.5 and 36 h reaction time	Time of addition of alcohol	0 h Two-step additions (supplemented at 0 h and 8 h, and the reaction continued up to 36 h). Three-step additions (supplemented at 0 h, 12 h, and 24 h, and the reaction continued up to 36 h)
Step 4	1000 mg biomass + molar ratio of acid/alcohol 1:1.5 addition of alcohol at 0 h	Time of reaction	6 h to 36 h

omass showed a cottony mass that turned from yellow to green over time (Fig. **1**).

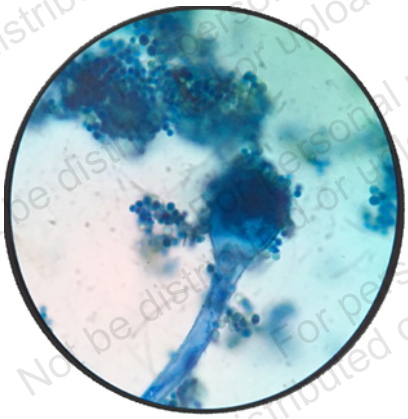

Fig. (1). Morphological characteristics of fungal culture RBD-01 (*Aspergillus flavus*). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.2. Effect of the Amount of Biomass

Variable amounts (500, 700, 1000, and 2000 mg) of biomass were used to determine the optimal levels that would facilitate the notable conversion of acid to esters. A maximum conversion (yield=48%) was observed when 1000 mg biomass was used in the reaction (Fig. **2**). Further increase in biomass extent to 2000 mg led to a decrease in the conversion of esters to 31%. A transformation of only 2% and 7% was also observed in the case of 500 mg and 700 mg of biomass, respectively (Fig. **2**). The optimized reaction condition suggested that an increase in the extent of biomass content beyond 1000 mg led to a decrease the product formation and reduced yield, which presumably was due to the increased viscosity of the reaction mixture in which homogeneous stirring could not take place. The observations on the excess biocatalyst not contributing to the increase in percentage conversion are further supported by the observations of Torres and Otero [15].

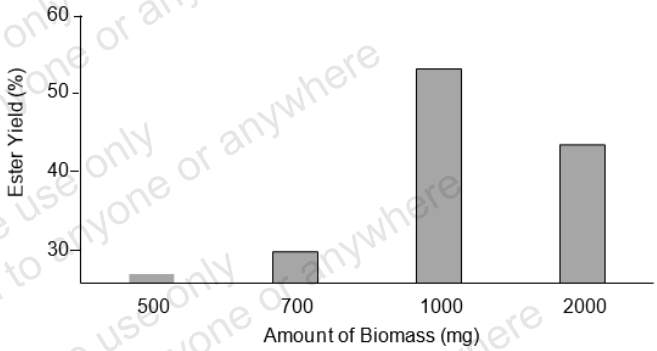

Fig. (2). Effect of biomass on esterification reaction.

3.3. Effect of Molar Ratio of Alcohol to Acid on the Esterification

The molar ratio of an acyl donor to an acyl acceptor plays a significant role in the esterification process. A maximum conversion of 52% was observed when propionic acid to ethyl alcohol molar ratio of 1:1.5 was used (Fig. **3**). The transformation of ester was observed to be 27%, 30%, and 7% in the case of 1:0.5, 1:1, and 1:2 acid/alcohol molar ratios, respectively (Fig. **3**). Higher concentrations of alcohol led to a decrease in the formation of ester due to the inhibition of enzyme activity. In general, the increased substrate concentration triggers product formation and accelerates the reaction rate [16]. The whole-cell catalyzed esterification reaction was greatly affected by the molar ratio of alcohol to acid concentration [3].

It was reported that, when added in excess, short-chain alcohols can modify the enzyme's hydrophilic end, resulting in denaturation [17]. Due to low molecular weight and higher polarity, methanol may easily diffuse and access the lipase enzyme localized in the cell membrane, thus catalyzing the reaction at a higher rate. However, the enzyme lipase is partially inactivated by the presence of an excessive amount of ethanol in the reaction system [17].

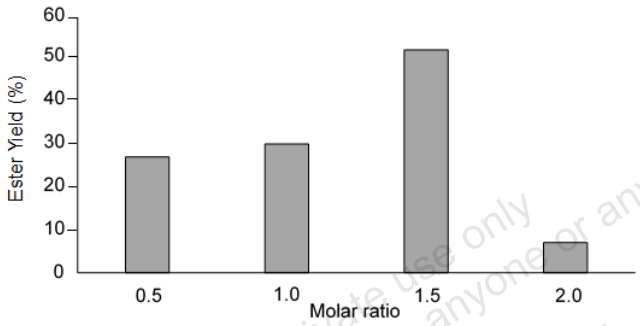

Fig. (3). Effect of molar ratio of acid/alcohol.

3.4. Effect of the Time of Addition of Alcohol

The time of alcohol addition also significantly influences the esterification reaction. Following the stepwise addition, alcohol was added in two-step and three-step intervals of 8 h and 12 h, respectively, to facilitate the reaction and determine the best yield of the esterification product.

One-time addition of alcohol at 0 h showed promising results with a yield of 52%. In two-step addition, alcohol was initially added at 0 h, followed by addition at 8 h, and the reaction was continued for 36 h. The final yield of the esterification product was only 2% compared to the one-time (0 h) addition of alcohol. In a similar pattern, a three-step addition, with a time interval from 8 h to 12 h between the successive additions of alcohol, did not enhance the ethyl ester yield. Comparatively, lower yields of products (3%) were obtained when the time interval of the addition of alcohol was 12 h, expectedly due to the reversible nature of the reaction. When the time interval of the addition of ethanol was greater than 0 h, although the alcohol was completely utilized in the reaction, the reaction was reversible, subsequently decreasing the product yield (Fig. **4**) [18].

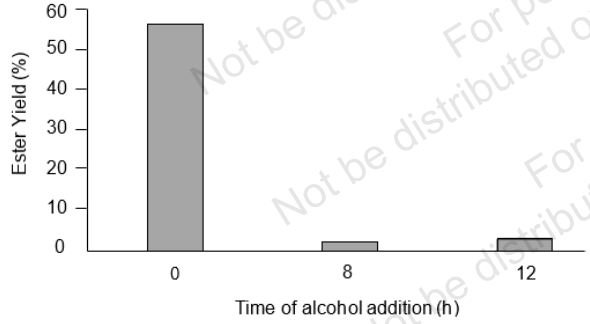

Fig. (4). Effect of time of addition of alcohol.

3.5. Rate of Reaction

In lipase-catalyzed reactions, the reaction time considerably influences the rate of reaction and product formation. The observations indicated that upon an increase in the reaction time, the extent of esterification increased up to 24 h, beyond which it noticeably decreased (Fig. **5**). The conversion percentage of ester increased from 2% at 6 h to 52% by 24 h. The reaction rate was thus directly dependent on the initial concentration of reactants and the time of reaction.

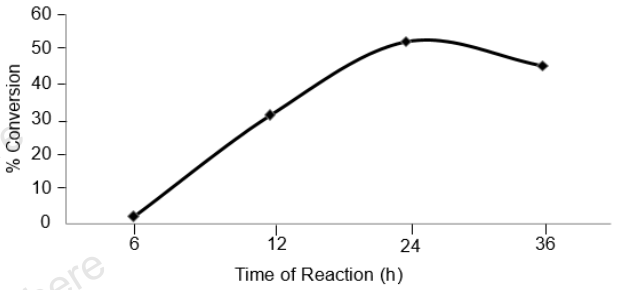

Fig. (5). Effect of time of reaction.

3.6. Other Aliphatic Acids as Acyl Donors

Based on the optimized conditions obtained with propionic acid and ethyl alcohol, the esterification reactions were further extended to a range of aliphatic acids as acyl donors and ethanol as an acyl acceptor using the dry biomass as the catalyst. The reaction was performed with 20 ml of the organic solvent containing 100 mM of each substrate (ethanol and aliphatic acid) in a 100 ml round bottom flask. To initiate the reaction, the whole cell catalyst was added to the reaction mixture, following the optimized conditions determined earlier, in an orbital shaking water bath at 37°C and 120 rpm for 72 h [19]. It was observed that the conversion of acid to esters was marginal in the case of short-chain aliphatic acids. The yield of ethyl propanoate (**1**) was about 5%, followed by ethyl butyrate (**2**) (2% yield) and ethyl pentanoate (**3**) (3% yield) (Fig. **6**). These esters, as reported previously [19], were highly volatile in nature. The yields of esters increased with the increasing chain length of acids, and yields of ethyl hexanoate (**4**), heptanoate (**5**), and octanoate (**6**) were 50%, 54%, and 50%, respectively. The conversion was observed maximum in the case of ethyl decanoate (84%) (**7**) (Fig. **6**). The yield of synthesized ethyl esters was calculated by analyzing the spectra obtained through NMR spectroscopy by applying the equation proposed by *Sharma et. al*. [14]. It was noted that the volatile nature of esters decreased with increasing the chain length of carbon (Table **2**). Yan Xu (2002) reported the synthesis of flavor ethyl esters of short-chain fatty acids using lipases wherein the whole cell and cell-free lipase from *Rhizopus chinensis* were used as catalysts for the esterification of hexanoic acid with ethyl alcohol. A maximum conversion of 96.5% was obtained in 72h in the case of whole-cell lipase as a catalyst and 84.9% yield in the case of cell-free lipase [20]. Abbas *et al*. (2003) reported using Mucor sp. lipase immobilized on Amberlite IRC 50 to synthesize aromatic esters of propionic, butyric, and caproic acids with methanol, ethanol, allyl butanol, isoamyl, geraniol, citronellol and farnesol alcohol used as acyl acceptors [21]. In addition, the change from fruity to waxy fragrance was evident with the change in the chain length (Table **2**).

Ethyl hexanoates (**4**) were further considered for the esterification reactions to synthesize diverse alkyl hexanoates (**4A-4J**) due to their significant fruity aroma. Esterification reaction was performed using hexanoic acid as the substrate and all primary alcohols (methanol - decanol) as acyl acceptors.

Table 2. The yield of ethyl esters from different acids and their nature of the aroma.

S. No.	Alkyl Esters	Ester Yield (%) (Mean ± SD)	Fragrance
1	Ethyl propanoate	5.0 ± 0.11	Strong ripe apple
2	Ethyl butyrate	2.0 ± 0.30	Fresh red apple
3	Ethyl pentanoate	3.0 ± 0.025	Aniseed
4	Ethyl hexanoate	50.0 ± 1.97	Ripe fruit berry
5	Ethyl heptonate	54.0 ± 1.54	Ripe grapes
6	Ethyl octanoate	50.0 ± 1.52	Coconut
7	Ethyl decanoate	84.0 ± 0.57	Oily

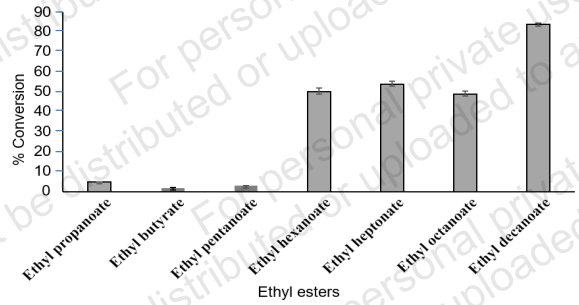

Fig. (6). Shows the comparison in ester yield from different acids with ethanol.

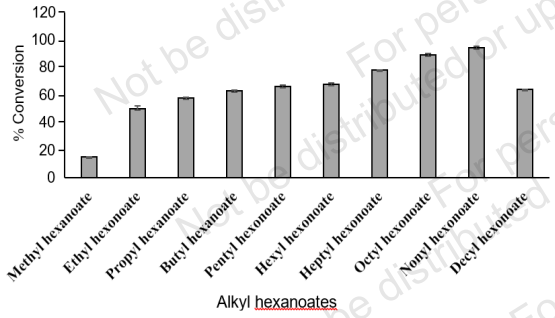

Fig. (7). Effect of chain length of alcohols on esterification of hexanoic acid.

3.7. Synthesis of Alkyl Hexanoates

The esterification reaction of hexanoic acid with all primary alcohols (methanol to decanol) was performed with lipase-catalyzed whole-cell biocatalyst to synthesize alkyl hexanoates (4A-4J) as per the previously outlined optimized conditions. The product formation was found to increase from methyl hexanoate (4A) to nonyl hexanoate (4I) with the increase in carbon chain length (Fig. **7**). However, decyl hexanoate (4J) was found to convert lesser than other alky hexanoates (Fig. **7**). The yield of alkyl hexanoates was quantified with the help of NMR spectroscopy as per the above-mentioned equation proposed by Sharma *et al*. Maximum conversion was observed when nonanol (94% yield) was used as an acyl acceptor (Table **3**).

Keeping in view the fragrance-like ripe fruit berry of ethyl hexanoate (4B), all synthesized alkyl hexanoates (4A-4J) were tested for their aroma quality. From the results mentioned in Table **3**, it was observed that except decyl hexanoate (4J), all synthesized compounds (4A-4I) exhibited fruity pineapple/berry flavors with significant fragrance.

Table 3. The yield of alkyl esters of different alcohols with hexanoic acid and their nature of the aroma.

S. No.	Alkyl Esters	Ester Yield (%) (Mean ± SD)	Fragrance
4A	Methyl Hexanoate	15.0 ± 0.54	Strong pineapple
4B	Ethyl Hexanoate	50.0 ± 1.97	Sweet pineapple
4C	Propayl Hexanoate	58.0 ± 0.32	Sweet tropical pineapple
4D	Butyl Hexanoate	62.0 ± 0.68	Fruity berry
4E	Pentayl Hexanoate	66.0 ± 1.46	Fruity green apple
4F	Hexayl Hexanoate	68.0 ± 1.02	Waxy, berry
4G	Heptayl Hexanoate	78.0 ± 0.27	Green sappy
4H	Octayl Hexanoate	89.0 ± 0.97	Coconut oily
4I	Nonayl Hexanoate	94.0 ± 1.57	Flowery
4J	Decayl Hexanoate	64.0 ± 0.06	Oily

CONCLUSION

In summary, we demonstrated the use of a whole-cell biocatalytic process with *Aspergillus flavus* (RBD-01), facilitating the conversion of aliphatic acids with ethanol to obtain a variety of important alkyl aroma esters. The study revealed that the major factors which could influence esterification reactions are the length of the carbon chain of substrate/alcohol, substrate concentration, stirring speed and reaction time, the molar ratio of alcohols, and the amount of biocatalyst. The ethyl hexanoate (4) possessed a fruity fragrance with a good conversion rate among all synthesized alkyl esters. Good yields were obtained with almost all of the aliphatic acids, with the yield of esters increasing with the chain length of alcohol examined. The synthesized alkyl hexanoates (4A-4I) were observed to possess good fruity/pineapple/berry fragrances with aroma quality. The demonstrated synthesis reactions can thus be considered for further exploration in wide industrial applications, especially in the food/cosmetic industries.

LIST OF ABBREVIATIONS

BHB = Bushnell Haas Broth

HRMS = High-resolution Mass Spectrum

PDA = Potato Dextrose Agar

PDB = Potato Dextrose Broth

TLC = Layer Chromatography

TMS = Tetramethyl Silane

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data and supportive information are available within the article.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

The 1H NMR spectral data of compounds **2-7** and **4A-4J** are provided in the supporting information section.

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