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DCC/DMAP mediated esterification of hydroxy and non-hydroxy olefinic fatty acids with β-sitosterol: *In vitro* antimicrobial activity

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Abstract

A new series of fatty alkenoates were synthesized using an appropriate synthetic route involving DCC and DMAP as catalysts. Compounds were characterized by their spectral data. All the synthesized compounds were evaluated for their *in vitro* antimicrobial activity. The minimum inhibitory concentration (MIC), minimum bacterial concentration (MBC) and minimum fungicidal concentration (MFC) were determined for test compounds as well as for reference standards. Among the compounds tested, compounds having hydroxy group at the fatty acid chain showed the most potent antibacterial as well as antifungal activities.
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Keywords: Fatty acids; β-Sitosterol; DCC; DMAP; IR; NMR; Mass; Antimicrobial activity

The negative health trends call for a new interest in infectious disease in the medical and public health communities and renewed strategies on treatment and prevention. Solutions outlined by the Centre for Disease Control and Prevention (CDC) include: prevention, (such as vaccination); improved monitoring; and the development of new treatments. Last solution would encompass the development of new antimicrobials by which these infectious diseases can be defeated [1]. Many seed oils, fatty acids and their derivatives are known for their antimicrobial [2], antifungal [3] and pesticidal [4] activities. A number of investigations have demonstrated that a variety of modified fatty acids are promising molecules in cancer prevention and have potential in the treatment of cancers [5,6]. Fatty alkenoates have received very little attention despite the fact that such molecules have been found to be associated with diverse biological activities such as antioxidant [7], antifeedant [8], anti-inflammatory [9], antiparasitic [10], antimicrobial [11] and neuroprotective [12]. Some fatty esters have been also found very effective for treatment of dermatitis [13], cardiovascular, hepatic and renal disorders [14]. Thus fatty alkenoates may lead to a new route to potential pharmaceutical molecules.

A number of methods [15] reported for esterification of fatty acids require either acidic or basic conditions or application of the heat. *N*,*N*-Dicyclohexylcarbodiimide (DCC) is used as a dehydrating/activating agent in organic synthesis [16] and is extensively used for building the peptide and amide linkages [17]. Literature reveals that long-chain fatty acids have not been esterified with β-sitosterol in the presence of DCC.

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The purpose of this study was to find the novel bioactivity of β-sitosteryl esters. It was discussed that β-sitosterol derivatives could have antimicrobial activity [18,19]. In view of the significance of long-chain fatty acids as potential pharmacophores, we report here the synthesis and spectral studies of new β-sitosterol analogs containing C11 and C18 fatty acids along with their *in vitro* evaluation against a panel of Gram-positive, Gram-negative strains of bacteria and some strains of fungus.

1. Experimental

Undec-10-enioc (purity 98%) and (*9Z*)-octadec-9-enoic (97%) acids were purchased from Fluka Chemicals (Buchs, Switzerland). (*9Z*,*12R*)-12-Hydroxyoctadec-9-enoic (ricinoleic) and (*9R*,*12Z*)-9-hydroxyoctadec-12-enoic (isoricinoleic) acids were isolated from the natural sources, i.e. from *Ricinus communis* and *Wrightia tinctoria* seed oils respectively. The concentrate of pure hydroxy acids was obtained by Gunstone's partitioning [20] of freshly prepared acids and further purified by column chromatography. β-Sitosterol was isolated from *Ficus krishnae* following the literature procedure [21].

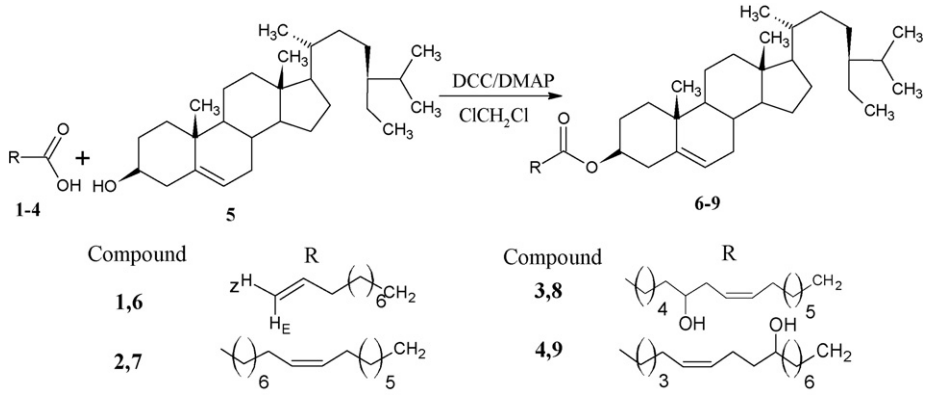
Fatty acid (5 mmol), DCC (5.5 mmol), and β-sitosterol (5 mmol) in dichloromethane (20 mL) with catalytic amount of 4-dimethylaminopyridine (DMAP) were stirred mechanically at room temperature until esterification was complete. *N*,*N*-dicyclohexylurea formed was filtered off. The filtrate was washed with water (3 × 20 mL), 5% acetic acid (3 × 20 mL) again with water (3 × 20 mL) and dried over anhydrous sodium sulphate. Solvent was removed under the reduced pressure to give the ester **6–9** (Scheme 1) which were chromatographed over a column of silica gel using *n*-hexane–ethyl acetate (96:4, v/v) as an eluent. All these newly synthesized compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR and mass spectral data [22].

The newly synthesized compounds were screened for their *in vitro* antibacterial activity against selected bacterial strains by disk diffusion method [23]. Chloramphenicol (30 μg) was used as positive control. While the disk poured in DMSO was used as negative control. The susceptibility was assessed on the basis of diameter of zone of inhibition against Gram-positive and Gram-negative strains of bacteria. Inhibition zones were measured and compared with the controls. The bacterial zones of inhibition values are given in Table 1. MICs and MBCs were determined by broth dilution technique and the results are given in Table 2.

Antifungal activity was also done by disk diffusion method. For assaying antifungal activity was measured by agar diffusion method [24]. The fungal activity of each compound was compared with greseofulvin as a standard drug. The fungal zones of inhibition values are given in Table 3. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) are given in Table 4.

2. Results and discussion

The use of catalysts has become popular among synthetic organic chemists for improving classical organic reactions, shortening reaction time and/or improving yields, as well as promoting new reactions. To explore the probability of getting the pharmacophoric important moiety from natural products in higher yields and in shorter



Scheme 1. Synthesis of fatty acid esters of β-sitosterol using DCC/DMAP as a catalyst.

Table 1
Antibacterial activity of the newly synthesized fatty alkenoates.

Compounds	Diameter of zone of inhibition (mm)			
	Gram-positive bacteria		Gram-negative bacteria	
	B. subtilis	*S. aureus*	*E. coli*	*S. typhimurium*
6	17.8 ± 0.4	17.3 ± 0.5	18.1 ± 0.6	16.3 ± 0.4
7	15.6 ± 0.3	15.2 ± 0.4	16.5 ± 0.2	14.6 ± 0.3
8	21.9 ± 0.8	20.6 ± 0.3	25.2 ± 0.3	21.7 ± 0.4
9	20.1 ± 0.2	20.2 ± 0.3	23.9 ± 0.2	18.9 ± 0.9
Standard	23.0 ± 0.2	22.0 ± 0.2	27.0 ± 0.2	19.0 ± 0.2
DMSO	–	–	–	–

Positive control (standard); chloramphenicol and negative control (DMSO) measured by the Halo Zone Test (unit, mm).

Table 2
MIC and MBC results of newly synthesized fatty alkenoates positive control chloramphenicol.

Compounds	Gram-positive bacteria				Gram-negative bacteria			
	B. subtilis		*S. aureus*		*E. coli*		*S. typhimurium*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
6	12.5	25	12.5	12.5	12.5	25	12.5	25
7	25	25	25	50	12.5	50	25	50
8	6.25	12.5	12.5	12.5	6.25	12.5	25	6.25
9	6.25	25	12.5	12.5	6.25	12.5	25	6.25
Standard	6.25	12.5	6.25	12.5	6.25	12.5	6.25	12.5

MIC (μg/mL), minimum inhibitory concentration, i.e. the lowest concentration of the compound to inhibit the growth of bacteria completely; MBC (μg/mL), minimum bacterial concentration, i.e. the lowest concentration of the compound for killing the bacterial completely.

Table 3
Antifungal activity of newly synthesized fatty alkenoates.

Compounds	Diameter of zone of inhibition (mm)			
	CA	AN	HO	PM
6	26.1 ± 0.3	23.1 ± 0.2	18.6 ± 0.4	17.3 ± 0.5
7	22.2 ± 0.4	20.6 ± 0.4	21.2 ± 0.2	16.2 ± 0.3
8	23.1 ± 0.7	22.3 ± 0.7	19.9 ± 0.6	15.1 ± 0.9
9	23.6 ± 0.4	20.8 ± 0.2	19.0 ± 0.3	15.2 ± 0.2
Standard	30.0 ± 0.2	27.0 ± 0.2	24.0 ± 0.3	20.0 ± 0.5
DMSO	–	–	–	–

Table 4
MIC and MFC results of newly synthesized fatty alkenoates positive control greseofulvin.

Compounds	CA		AN		HO		PM	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
6	12.5	25	25	50	25	50	25	100
7	12.5	25	12.5	25	12.5	25	12.5	50
8	6.25	25	6.25	25	12.5	25	25	50
9	12.5	50	25	50	25	50	25	100
Standard	6.25	12.5	6.25	12.5	6.25	12.5	6.25	12.5

CA, *Candida albicans*; AF, *Aspergillus niger*; HO, *Heliminthosporum oryazae*; PM, *Penicillium marneffei*. MIC (μg/mL), minimum inhibitory concentration, i.e. the lowest concentration of the compound to inhibit the growth of fungus completely; MFC (μg/mL), minimum fungicidal concentration, i.e. the lowest concentration of the compound for killing the fungus completely.

reaction time, our attention has turned to employ the use of catalysts. The use of catalysts for the synthesis of fatty alkenoates provided higher yield of products. In most of the cases the yield of product was 74–88%. At first the esterification of β-sitosterol with undec-10-enoic acid was chosen as a model to optimize the conditions for the preparation of compounds **6–9**. In order to determine the optimum conditions for the synthesis fatty alkenoates, variations in molar ratios of reagents and the catalysts were investigated. After some experimentation, we found a set of conditions that generally provide products in good yield and thus optimum conditions for molar ratio of fatty acids, β-sitosterol, DCC and DMAP were set up. The generality and scope of the synthetic procedures were demonstrated by subjecting β-sitosterol with olefinic (terminal and internal) and hydroxy olefinic carboxylic acids. IR absorptions characteristic of ester (1732–1737 cm^{-1}) were observed in all the newly synthesized compounds.

In the ^1H NMR spectra of compound **6**, the olefinic protons were observed at δ_H 5.80 (tdd, 1H, $J_{H-9'CH_2} = 6.6$ Hz, $J_{H-H_Z} = 10.2$ Hz, $J_{H-H_E} = 16.4$ Hz, CH_2=C*H*–), 5.38 (d, 1H, $J = 4.4$ Hz, C_6–H), 4.95 (dd, 1H, $J_{H_Z-H} = 10.2$ Hz, $J_{H_Z-H_E} = 2.6$ Hz, H_ZC=CH), 4.91 (dd, 1H, $J_{H_E-H} = 16.4$ Hz, $J_{H_E-H_Z} = 2.8$ Hz, H_EC=CH–) and correlated with observations at δ_C 114.23, 122.67, 139.77, 139.27 respectively. A multiplet at δ_H 4.62 was observed for $C_3\beta$-H and correlated with observation at δ_C 73.75. Besides few other significant carbon signals for the fatty acid chain characteristic peak at δ_C 174.41 ($C_{1'}$, ester C=O) were recorded. Spectral studies have illustrated that the change in the nature of fatty acids at β-C_3 has not significantly influenced the pattern of proton and carbon signals of the β-sitosterol moiety.

The investigation of antibacterial screening data revealed that all the tested compounds showed moderate to good bacterial inhibition. In most of the cases compounds **8** and **9** showed good antibacterial activity nearly equivalent to that of chloramphenicol. The MBC of few compounds was found to be the same as MIC but in most of the compounds it was two or three folds higher than the corresponding MIC results. The antifungal screening data showed only moderate activity. Among the screened compounds, **6** showed good inhibition against *Candida albicans*. Compounds **8** and **9** revealed good results against *C. albicans* and *Heliminthosporum oryazae* and moderate activity against *Aspergillus niger* and *Penicillium marneffei*. The MFC of all the compounds was two or three folds higher than the corresponding MIC results.

The varied divergence in the antimicrobial activity of these compounds validates the reason of this study. The importance of such kind of work lies in the possibility that the new compounds might be more efficient against bacteria and a thorough study regarding the structure–activity relationship, toxicity and their biological effects would be helpful in designing more effective antimicrobial agents for therapeutic use.

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[22] Selected spectroscopic data: 3β-sitosterrylundec-10′-enoate **6**: White solid; mp 93 °C, *Rf* = 0.76 (*n*-hexane/ethyl acetate, 3:2, v/v, as developer), isolated yield, 88%. IR (KBr, cm^{-1}): 2931, 2860, 1736; ^1H NMR ($CDCl_3$): δ_H 5.80 (tdd, 1H, $J_{H-9'CH_2} = 6.6$ Hz, $J_{H-H_Z} = 10.2$ Hz, $J_{H-H_E} = 16.4$ Hz, $CH_2{=}CH{-}$), 5.38 (d, 1H, $J = 4.4$ Hz, C_6–H), 4.95 (dd, 1H, $J_{H_Z-H} = 10.2$ Hz, $J_{H_Z-H_E} = 2.6$ Hz, $H_ZC{=}CH$), 4.91 (dd, 1H, $J_{H_E-H} = 16.4$ Hz, $J_{H_E-H_Z} = 2.8$ Hz, $H_EC{=}CH{-}$), 4.62 (m, 1H, C_3β–H), 2.31–1.07 (*br m*, 46H), 1.01 (s, 3H, C_{10}–CH_3), 0.92 (d, 3H, $J = 6.7$ Hz, C_{20}–CH_3), 0.86 (dd, 6H, $J = 6.4$ Hz C_{25}–$(CH_3)_2$), 0.84 (d, 3H, $J = 6.4$ Hz, C_{29}–CH_3), 0.68 (s, 3H, C_{13}–CH_3). ^{13}C NMR ($CDCl_3$): δ_C 173.41, 139.77, 139.27, 122.67, 114.23, 73.75, 56.74, 56.17, 50.06, 42.37, 39.77, 39.59, 38.22, 37.78, 37.06, 36.66, 36.25,35.88, 34.78, 33.89, 31.97, 31.91, 29.38, 29.30, 29.17, 29.15, 28.97, 28.32, 28.10, 27.88, 25.13, 24.36, 23.90, 22.92, 22.66, 21.10, 19.41, 18.79, 14.9, 11.93; ESI-MS found $[M+Na]^+$ 603.90; $C_{40}H_{68}O_2$ $[M+Na]^+$ requires 603.83.
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