Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(11):284-289

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Synthesis of *N*- Glycolipidamides with Antifungal and Surfactant Properties

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ABSTRACT

Synthesised N- glycosyl amide molecules of fatty acids with carbon chain lengths varying from C6 to C18 via the modified Staudinger reaction. Biosurfactant properties of these non-ionic compounds were determined along with their antifungal activity. The glucolinoleiamide (5e) and maltolinoleiamide (5f) have exhibited good surface tension with 37 mN/m and 43 mN/m respectively which are comparable with that of rhamnolipids. Compound 5e also showed very good fungicidal activity against Sclerotium rolfsii (ATCC 62666) while maltolinoleiamide (5f) and glucohexanoylamide (5d) exhibited inhibitory activity against Mucor indicus (MTCC 7135).

Keywords: Staudinger ligation; N-Gycosylamides; Rhamnolipid; Surfactants

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INTRODUCTION

The amide bond functionality plays a major role in the elaboration and composition many important natural and synthetic compounds [1,2]. Most known biosurfactants are glycolipids where carbohydrates are combined with long-chain aliphatic acids or hydroxyaliphatic acids via amide bond. The formation of amides between carboxylic acids and amines having steric hindrance or low reactivity can be accomplished by using coupling reagents or at high temperatures [3,4]. It is necessary to first activate the carboxylic acid, a process that usually consists of converting the –OH of the acid into a good leaving group prior to treatment with the amine [5]. The most common methods involve either conversion of carboxylic acid to a more active functional group such as acid chlorides, mixed anhydrides, esters, or via insitu activation of carboxylic group [6,7]. But it is also important to activate sugars by converting into azides before coupling with acid counterparts. Thus azides became an important tool especially for the synthesis of glycopeptides and post translational modification of proteins [8, 9]. Staudinger-type reactions offer a convenient access to prepare N-glucoproteins where the glycan chains are attached to the protein via β-glycosyl amide [10-12] using generally triphenylphosphine. In order to avoid triphenylphosphine impurities, Czifrak et al., replaced it with trialkyl analog to synthesize N-(β-D-glucopyranosyl) monoamide of dicarboxylates which are potential inhibitors glycogen phosphorylase [13]. Amphiphilic glycosylamides which are non-ionic biosurfactants of glycosyl amides have been prepared via Staudinger reaction in presence of trimethylphosphine [14-16]. The Staudinger reaction could allow the direct reaction of carboxylic acids or their halides, anhydrides or esters with glycosyl azides without transient reduction [17-19].

In the present study, we have successfully made iminophosphoanes of glycosylazides and reacted with triphenylphosphine/NBS activated alkanoic acids of C6 to C18 carbons to get *N*-glycolopidamides 5a-5f.

EXPERIMENTAL SECTION

Characterisation of compounds

Melting points were recorded on an Acro melting point apparatus using a calibrated thermometer. Thin layer chromatography (TLC) and column chromatography (CC) were performed with silica gel. [TLC silica gel 60

F254, Merck] [CC Kieselgel 60, 230-400 mesh,Merck] . Chromatograms were developed using hexane-EtOAc (8:2, v/v) and chloroform-MeOH (9:1, v/v). IR spectra were recorded on Thermo-Nicolet instrument in KBr discs. Mass spectra were recorded using GCMS-QP2010S (direct probe). NMR spectra were recorded in Jeol 400MHz and Bruker 400MHz in DMSO-d6 with TMS (tetra methyl silane) as an internal standard and chemical shifts were given in δ units.

Surfactant properties

Determination of emulsification index:

A mixture of 5 ml solution (50 mg compound in 5ml of water) and 5 mL kerosene (or diesel) was vertically stirred for 2 min and the height of the emulsion layer was measured after 24 h to determine the emulsification index [20]. The results were calculated and tabulated as in table 1. A higher emulsification index indicated a higher emulsification activity of the test compounds.

The equation used to determine the emulsification index (EI_{24} (%)) is as follows:

$$\% EI_{24} = \frac{\text{The height of emulsion layer}}{\text{The height of total solution}} \times 100$$

Surface tension: Measurement of the Critical Micelle Concentration (CMC):

CMC is an important parameter during the evaluation of biosurfactant activity. The surface tension of the aqueous solution at different surfactant concentrations was measured by the Du-Nouy ring method with a Kruss Tensiometer (Kruss, Hamburg, Germany) [21].The surface tension measurement was carried out at 25±1°C after dipping the platinum ring in the solution for a while in order to attain equilibrium conditions. The CMC was then determined from the break point of the surface tension versus log of bulk concentration curve. The surface tension of a surfactant reaches the lowest value at its CMC. Above this concentration, no further effect can be observed on the surface activity. For the calibration of the instrument, the surface tension of the pure water was measured before each set of experiments. The measurement was repeated three times and an average value was obtained.

Oil displacement method:

The oil displacement test is a method used to determine the surface activity by measuring the diameter of the clear zone, which occurs after dropping a surfactant-containing solution on a thin layer of oil on water. The binomial diameter allows an evaluation of the surface tension reduction efficiency of a test compound [22]. 15 µL of crude oil is placed on the surface of distilled water placed in a petri dish (90mmx 15mm) and then 10 µl of each test solution was slowly dropped on the surface of the oil film. The test was conducted at room temperature (25-27°C). The maximum diameter of the clear zone was observed under light and measured. The larger the diameter of the clear zone, the higher the surface activity of the test solution. Results were tabulated as in table 1.

Hydrophilic-Lipophilic Balance (HLB value):

Griffin's method for non-ionic surfactants is a measure of the degree to which it is hydrophilic or lipophilic, determined by calculating values for the different regions of the molecule. HLB scale indicates classification of surfactant function values are presented in table 1. It can be calculated by following equation

HLB = 20 * MWHP/MWSA

(MWHP indicates the molecular weight of the hydrophilic part and MWSA indicates the molecular weight of the whole surface-active agent)

Antifungal assays against pathogenic fungi

All compounds were assayed *in-vitro* for their activity against fungal pathogens viz., *Mucor indicus* (MTCC 7135), *Aspergillus niger* (MTCC 9687), *Fusarium oxysporum* (MTCC 2087), *Pythium aphanidermatum* (MTCC 10247), *Phytophthora infestans* (ATCC 64099), *Sclerotium rolfsii* (ATCC 62666) through poison plate method. Known quantity of the compounds were mixed to get the test concentrations with media Potato Dextrose Agar - Carrot agar media (PDA, CA, HiMedia) and poured into plates. All the experiments were performed in triplicates. A 5mm paper disc with test fungus from a fresh culture was inoculated in poison plate and incubated at 28 ± 1°C for 5 days. The average colony diameter was measured 4 times across the colony. Percentage growth inhibition was measured and compared with untreated control for all fungi and MIC values were calculated (Table 2).

RESULTS AND DISCUSSION

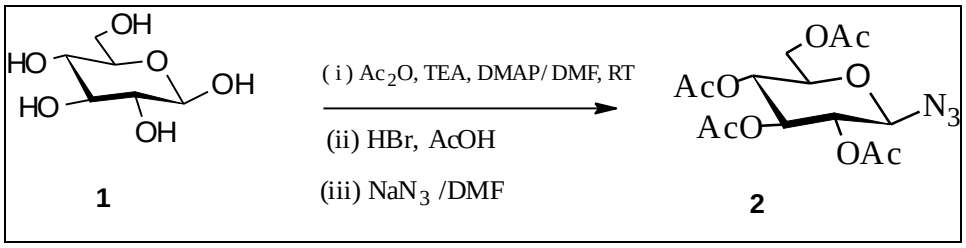
Chemistry

Synthesised several mono and disaccharide glycoamides by utilizing the modified Staudinger methodology. The key step in the synthesis of the glycopyranosyl conjugates is the coupling of a glycosylazide with activated acid moieties in the presence of triphenylphosphine. The classical Staudinger reaction is a two-step process involving the initial electrophilic addition of an azide to a trialkyl or triaryl phosphine followed by nitrogen elimination from the intermediate phosphazide to give the iminophosphorane. Usually, the imination proceeds smoothly, almost quantitatively, without the formation of any side products.

Synthesis of glycosyl amides using different long chain fatty acids:

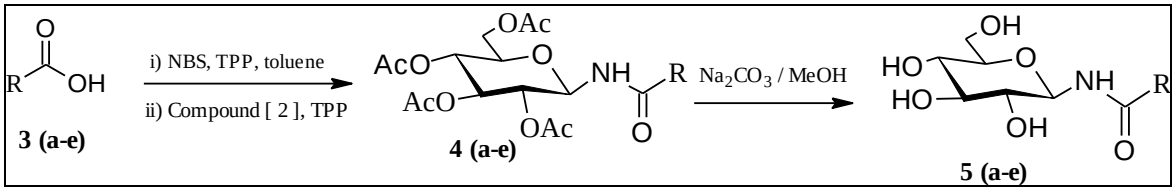
Glycosyl amides were synthesised via Staudinger ligation method in two steps.

Step 1: Glucose (1) was converted into its azide by protecting all hydoxy groups into their acetates using acetic anhydride in presence of TEA and DMAP in DMF at room temperature. α-Bromination for glycosyl peracetate carried out using HBr in acetic acid to obtain aceto bromo-α-D-glucose, which was treated with sodium azide in DMF to get acetylglucosylazide (2). In a similar way acetylmaltosylazide (2a) was made for preparation of compound 5f.

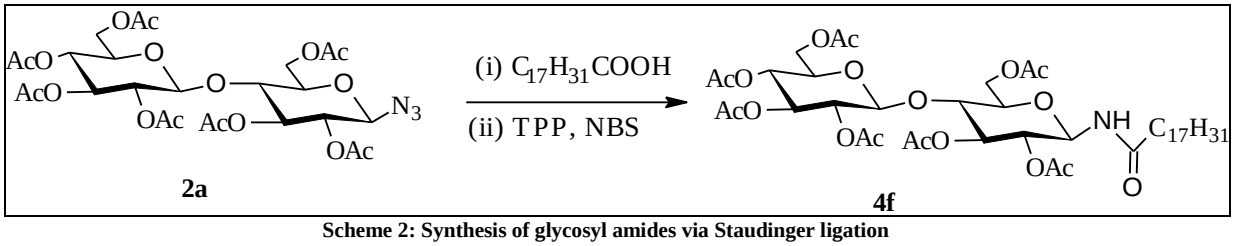


Scheme 1: Synthesis of glycosyl azide

Step 2: Glycosyl iminophosphoranes were reacted with pretreated acids 3 (a-e) with triphenyl phosphene and NBS to get acetylated amides 4 (a-e) and 4f from 2a. These were purified by column chromatography over silicagel. These products were deacetylated using sodium carbonate in methanol to get amides 5 (a-f) as shown in figure 1.



Where, R is a: Dodecanoic; b: Decanoic; c: Octanoic; d: Hexanoic; e: Linoleic



Scheme 2: Synthesis of glycosyl amides via Staudinger ligation

General Procedure for the preparation of Glycolipidamides (5a-5f)

To a stirred solution of long chain fatty acid (0.025 mol) and toluene (50 mL), added triphenylphosphine (0.05mol, 2eq) at room temperature. To this reaction mixture added NBS (0.05 mol, 2 eq) in portion wise under cooling condition (0-5^{o}C) and maintained at room temperature for 1h. Then added glycosyl iminophosphoranes (0.0275mol, 1.1 eq) and reaction mixture was stirred at room temprature for 1.5h. The completion of the reaction was monitored by TLC. The reaction mass was diluted with EtOAc (200 mL) and washed with 5% aqueous $NaHCO_3$ solution (2X100 mL). Resulting organic layer was concentrated to provide crude compounds, which was further purified by using silica-gel column chromatography by eluting with mixture of hexane: EtOAc to give pure compounds 4 (a-e).

To a stirred solution of compound (0.019 mol) in MeOH (130 mL) was added sodium carbonate (0.057 mol, 3eq) at room temperature and maintained at the same temperature for 1.5 h. The completion of the reaction was monitored by TLC. The reaction mixture was filtered to remove sodium carbonate and organic layer was concentrated to give crude compounds 5a-5e, which were further purified by using silica-gel column chromatography by eluting with mixture of DCM and MeOH to get pure compounds 5a-5f (Yield: ~65-70%).

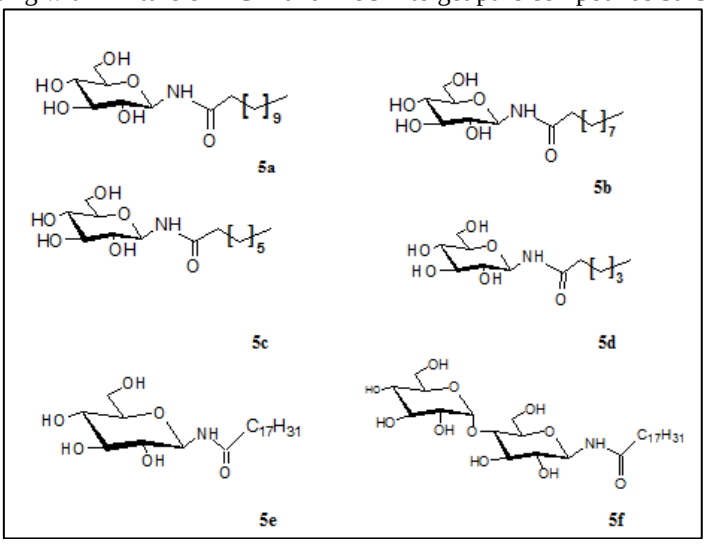


Figure 1: Synthesized Glycolipidamides 5a-5f

5a. N-Dodecanoyl-β-D-glucopyronosylamine

White solid; $C_{18}H_{35}NO_6$; $[\alpha]_D$ +12° (*c* 1, Me_2SO); MP: 175-178°C.

IR (KBr): ν_{max} 3325, 2919, 1634, 1548, 1351, 1251, 1081, 891 cm^{-1}

^{1}H-NMR (DMSO-d6, 400MHz); δ 0.85 (3H, t, *J*=6.8 Hz, CH_3), 1.24 (16H, bs, 8XCH_2), 1.47 (2H, m), 2.05-2.09 (2H, m), 3.00-3.09 (3H, m),3.16 (1H,t, *J*=8.4Hz), 3.63 (1H, dd, *J*=11.6 Hz), 4.48 (1H, brs), 4.69 (1H, dd, *J*=9.2 Hz), 8.26 (1H, d, J=9.2 Hz).

^{13}C-NMR (DMSO-d6, 100 MHz): δ 172.60 (NH-C=O), 79.46, 78.49, 77.59, 72.48, 70.02, 60.96, 35.47, 31.35, 29.11, 29.07, 29.01, 28.93, 28.83, 28.77, 24.94, 22.15, 13.99.

GC-MS (*m/z*): 361 [$M^{+\cdot}$] (10), 343 (5), 330 (5), 312 (5), 292 (4), 282 (12), 270 (15), 254 (3), 240 (20), 228 (50), 210 (28), 200 (85), 183 (100), 162 (32), 144 (25), 126 (14), 116 (15).

5b. N-Decanoyl-β-D-glucopyronosylamine

White solid; $C_{16}H_{31}NO_6$; $[\alpha]_D$ +19° (*c* 1, Me_2SO); MP: 181-183°C.

IR (KBr): ν_{max} 3323, 2919, 1633, 1549, 1352, 1265, 1082, 891 cm^{-1}

^{1}H-NMR (DMSO-d6, 400MHz); δ 0.85 (3H, t, *J*=6.8 Hz, CH_3), 1.24 (12H, brs, 6XCH_2), 1.47 (2H, brs), 2.07 (2H, m), 3.03-3.05 (3H,m), 3.16 (1H,m), 3.63 (1H, dd, *J*=11.2 Hz), 4.48 (1H, brs), 4.68 (1H, dd, *J*=9.2 Hz), 8.28 (1H, d, *J*=8.4 Hz).

^{13}C-NMR (DMSO-d6, 100 MHz): δ 172.65 (NH-C=O), 79.48, 78.50, 77.58, 72.46, 70.03, 60.97, 35.47, 31.35, 28.96, 28.94, 28.83, 28.76, 24.95, 22.16, 13.02.

GC-MS (*m/z*): 333 [$M^{+\cdot}$] (10), 316 (15), 302 (5), 284 (5), 267 (4), 254 (3), 242 (22), 226 (50), 212 (28), 200 (45), 182 (20), 172 (62), 155 (100), 144 (14), 129 (10), 116 (15).

5c. N-Octanoyl-β-D-glucopyronosylamine

White solid; $C_{14}H_{27}NO_6$; $[\alpha]_D$ + 9.2° (*c* 1, Me_2SO); MP: 114-116°C.

IR (KBr): 3323, 2919, 1633, 1550, 1353, 1268, 1081, 890, cm^{-1}

^{1}H-NMR (DMSO-d6, 400MHz); δ 0.85 (3H, t, *J*=6.8 Hz, CH_3), 1.24 (8H, brs, 4XCH_2), 1.47 (2H, m), 2.03-2.11 (2H, m), 3.05-3.06 (3H, m), 3.16 (1H, t, *J*=8.4Hz), 3.61 (1H, dd, *J*=12.0 Hz), 4.53 (1H, brs), 4.67 (1H, dd, *J*=9.2 Hz), 8.41 (1H, d, *J*=8.4 Hz).

^{13}C-NMR (DMSO-d6, 100 MHz): δ 172.76 (NH-C=O), 79.56, 78.48, 77.51, 72.32, 70.00, 60.96, 35.44, 31.21, 28.77, 28.56, 24.95, 22.11, 13.99.

GC-MS (*m/z*): 305 [$M^{+\cdot}$] (10), 292 (15), 302 (5), 284 (5), 267 (4), 254 (3), 242 (22), 226 (50), 212 (28), 200 (45), 182 (20), 172 (62), 155 (100), 144 (14), 129 (10), 116 (15).

5d. N-Hexanoyl-β-D-glucopyronosylamine

White solid; $C_{12}H_{23}NO_6$; $[\alpha]_D$ +14.2° (*c* 1, Me_2SO); MP: 84-87°C.

IR (KBr): ν_{max} 3335, 2957, 1661, 1555, 1353, 1262, 1080, 882 cm^{-1}

^{1}H-NMR (DMSO-d6, 400MHz); δ 0.86 (3H, t, *J*=6.8 Hz, CH_3), 1.25 (4H, brs, $2XCH_2$), 1.48 (2H, m), 2.05-2.10 (2H, m), 3.03-3.05 (3H, m), 3.15 (1H, m), 3. 62 (1H, dd, *J*=11.6 Hz), 4.54 (1H, brs), 4.67 (1H, dd, *J*=9.2 Hz), 8.46 (1H, d, *J*=8.8 Hz).
^{13}C-NMR (DMSO-d6, 100 MHz): δ 172.74 (NH-C=O), 79.59, 78.50, 77.51, 72.29, 70.00, 60.96, 35.39, 31.02, 24.62, 21.96, 13.90.
GC-MS (*m/z)*: 277 [$M^{+\cdot}$] (12), 203 (5), 186 (5), 180 (7), 156 (5), 148 (15), 140 (18), 129 (8),116 (12), 99 (35).

5e. N-linoleoyl-β-D-glucopyronosylamine
White solid; $C_{24}H_{43}NO_6$; $[\alpha]_D$ +16° (*c* 0.5, Me_2SO); MP: 141-143°C.
IR (KBr): ν_{max} 3387, 2925, 1636, 1551, 1466, 1371, 1085, 825 cm^{-1}
^{1}H-NMR (DMSO-d6, 400MHz); δ 0.85 (3H, t, *J*=6.0Hz,CH_3), 1.26 (14H, brs, $7XCH_2$), 1.52 (2H, brs), 2.10-2.70 (8H, m), 3.20-3.40 (3H, m), 3. 62 (1H, m), 3. 72 (1H, m), 4.20 (1H, m), 4.85 (3H, m), 5.25 (2H, m), 7.62 (1H, d, *J*=8.8 Hz).
^{13}C-NMR (DMSO-d6, 100 MHz): δ 177.29 (NH-C=O), 130.97, 130.92, 129.07, 102.92, 81.05, 81.00, 78.87, 78.26, 75.11, 74.81, 74.23, 73.57, 71.59, 62.77, 62.11, 37.19, 32.67, 30.77, 30.48, 30.42, 30.37, 30.27, 28.20.
GC-MS (*m/z)*: 441 [$M^{+\cdot}$] (5), 423 (5), 410 (3), 392 (6), 380 (5), 362(8), 344 (6), 320 (28), 303(7), 290 (14), 279 (12), 263 (24), 245 (8), 180 (14), 162 (38), 144 (22), 135 (12), 121 (14).

5f. N-linoleoyl- 4-O-α- D-glucopyronosyl-1-β-D-glucopyronosylamine
White solid; $C_{30}H_{53}NO_{11}$; $[\alpha]_D$ +22.4° (*c* 0.5, Me_2SO); MP: 155-157°C
IR (KBr): ν_{max} 3386, 2921, 1634, 1466, 1548, 1462, 1080, 845 cm^{-1}
^{1}H-NMR (DMSO-d6, 400MHz); δ 0.86 (3H, t, *J*=6.0 & 7.6 Hz, CH_3), 1.25-1.33 (14H, m $7XCH_2$), 1.47 (2H, m), 1.99-2.04 (4H, m), 2.06- 2.10 (2H, m), 2.73 (2H, t, *J*=6.0Hz), 3.10-3.14 (1H, m), 3.16 (2H, brs), 3.25-3.32 (4H, m), 3.43-3.69 (4H, m), 4.21 (1H, d, *J*=7.2Hz), 4.72 (2H, t, *J*=8.4&9.2Hz), 5.26- 5.38 (4H, m), 8.50 (1H, d, *J*=8.4Hz).
^{13}C-NMR (DMSO-d6, 100 MHz): δ 177.78 (NH-C=O), 129.76(2XC), 127.77(2XC), 103.85, 80.62, 79.35, 76.40, 75.69, 75.57, 73.24, 71.96, 70.62, 68.11, 60.39, 35.40, 30.92, 29.09, 28.80(2xC), 28.74(2xC), 28.63, 26.69, 26.62, 25.24, 24.95, 22.00, 13.96.
GC-MS (*m/z)*: 603 [$M^{+\cdot}$] (5), 549 (4), 387 (3), 369 (5), 351 (4), 331 (7), 312 (25), 290 (5), 262 (5), 228 (5), 211 (4), 186 (5), 169 (60), 157 (5), 141 (10), 127 (14).

Surfactant properties

Table 1: Surfactant properties of synthesised glycolipidamides

Sl No	Oil spread	HLB test	[a]Surface tension average in γ (N/m) at 24°C	Emulsification index % (EI24)
5a	+	7.38	63±0.09	48.9
5b	+	9.77	56±0.06	45.3
5c	+	10.67	48±0.11	50.7
5d	++	11.75	45±0.07	51
5e	++	9.01	37±0.03	54.8
5f	++	10.26	43±0.09	56
Water	-	-	71±0.18	-
Rhamnolipid	+++	-	35±0.09	59
1% SDS	++++	-	38±0.05	51.8
(-ve control)	Kerosene	-	30±0.08	0
(+ve control)	Kerosene+Tween80	-	-	66.33

Oil spread method: '+' clear zone diameter 0.5-0.9 cm; '++' clear zone diameter 1.0-1.5 cm; '+++' clear zone diameter 1.5-2.1cm; '++++' clear zone diameter < 3.0cm; [a]Values expressed in mean ± SD.

Biology

Table 2: Antifungal activity of Glycolipidamides

Compounds	MIC in ppm											
	Sclerotium	% inhibition	Mucor	% inhibition	Fusarium	% inhibition	Phytopthora	% inhibition	Pythium	% inhibition	Aspergillus	% inhibition
5a	20	30.7	20	15.6	70	15.6	50	16.7	10	28	100	25.9
5b	20	33.3	50	13.3	70	13.3	20	13.3	10	32	50	27.8
5c	20	21. 3	20	17.8	70	15.6	50	11.7	10	24	50	20.3
5d	15	41	15	64.2	15	29.2	50	19	15	28.5	15	41.7
5e	15	67.9	20	42	50	33.9	50	15.2	15	31.3	15	44.8
5f	15	50.1	15	54.3	15	35.4	50	21	15	41.2	15	51.4
Ridomil	15	35.9	15	36	50	33.9	15	62.8	15	12.5	15	44.8

CONCLUSION

In conclusion, sugar-derived N-alkylamides constitute an interesting group of non-ionic surfactants which exhibited in general a good surface-activity. Compounds **5e** and **5f** exhibited good surface tension while compound **5f** showed good emulsification index comparable to that of rhamnolipids. Compound **5e** also showed very good fungicidal activity against *Sclerotium rolfsii* (ATCC 62666) while glucohexanoylamide **5d** and maltolinoleiamide **5f** exhibited inhibitory activity against *Mucor indicus* (MTCC 7135). It may be concluded that compounds with longer alkyl chains appear to be good antifungals and emulsifiers.

Acknowledgements
Authors wish to thank Dr.B.N.Manjunath for technical discussions , Dr.Anil Kush, CEO of Vittal Mallya Scientific Research Foundation, for his interest and Dr.A.C Karunakara, Head-QC for analytical data. A grant from Department of Biotechnology, India (Grant No. BT/PR4667/PID/6/634/2012) is gratefully acknowledged.

REFERENCES

[1] J Magano; GA Weisenburger, *Org Process Res Dev.*, **2016**, 20 (2), 140-177.
[2] E Valeur; M Bradley, *Chem. Soc. Rev.*, **2009**, 38(2), 606–631.
[3] BS Jursic; Z Zdravkovski, *Synthetic Commun.*, **1993**, 23(19), 2761-2770.
[4] RM Lawrence; SA Biller; OM Fryszman, *Synthesis.*, **1997**, 5, 553-558.
[5] L Perreux, A Loupy, F. Volatron, *Tetrahedron*. **2002**, 58, 2155-2162.
[6] A Christian; GN Montalbetti; V Falque, *Tetrahedron.*, **2005**, 61, 10827-10852.
[7] J Burés; M Martín; F Urpí; J Vilarrasa, *J. Org. Chem.*, **2009**, 74(5), 2203-2206.
[8] Z Györgydeák; J Thiem, *Adv. Carbohydr. Chem. Biochem.*, **2006**, 60, 103-182.
[9] HSG Beckmann, V Wittmann. Organic Azides: Syntheses and Applications, John Wiley & Sons, Chichester, **2010**, 469-490.
[10] G José; F Bolaños; Ó López, *Top. Heterocycl. Chem.*, **2007**, 7, 31-66.
[11] SS van Berkel; MB,van Eldijk; JC van Hest, *Angew. Chem. Int. Ed. Engl.*, **2011**, 50(38), 8806-8827.
[12] SK Hait; SP Moulik, *Curr. Sci.*, **2002**, 82 (9), 1101-1111.
[13] Czifrák; Z Hadady; T Docsa; P Gergely; J Schmidt; L Wess Johann; L. Somsák, *Carbohydr. Res.*, **2006**, 341(8), 947-956.
[14] P Boullanger; V Maunier; D Lafont, *Carbohydr. Res.*, **2000**, 324 (2), 97–106.
[15] FL Lin; HM Hoyt; HV Halbeek; RG Bergman; CR Bertozzi, *J. Am. Chem. Soc.*, **2005**, 127 (8), 2686-2695.
[16] M Köhn; R Breinbauer, *Angew. Chem. Int. Ed. Engl.*, **2004**, 43(24), 3106-3116.
[17] H Staudinger; J Meyer, *Helv. Chim. Acta*. **1919**, 2 (1), 635-646.
[18] YG Gololobov; LF Kasukhin, *Tetrahedron.*, **1992**, 48, 1353-1406.
[19] MB Soellner; BL Nilsson; RT Raines, *J. Am. Chem Soc.*, **2006**, 128(27), 8820-8828.
[20] GC David; GC Beena, *Appl. Environ. Microbiol.*,**1987**, 53(2), 224-229.
[21] K Lunkenheimer; K D Wantke, *Adv. Colloid Interface Sci.* **1978**, 66(3), 579-581.
[22] M Morikawa; Y Hirata; T. Imanaka, *Biochim Biophys Acta*., **2000**, 1488 (3), 211-218.