CHAPTER - II

STUDY OF ENZYME CATALYSED REACTIONS

CHAPTER – 2

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INTRODUCTION

In the Chapter – I, the remarkable biosynthetic ability of Rhizopus arrhizus has been discussed. This prompted us to study and explore the utility of R. arrhizus for the biotransformation of various substrates with the carbonyl functionalities. This chapter deals with the preparation of the substrates used and the study of enzyme catalyzed reactions of these substrates. We have prepared β-Benzoyl propionic acid, 3-methyl-β-benzoyl propionic acid, p-methoxy-benzoyl-2-propionic acid, 3-Benzoyl-2-propenoic acid by using Friedel Crafts reaction [1]. The esters of above acids were also prepared. 3-Nitro-4-methyl acetophenone was prepared by the nitration of 4-methyl acetophenone as given in literature [2].

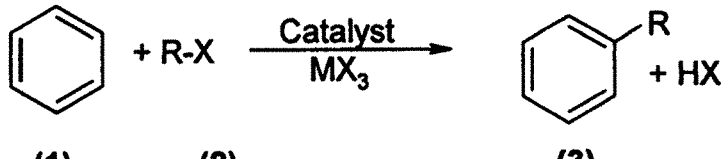
The Friedel – Crafts Reaction [3]

Friedel-craft reaction is usually associated with the alkylation or acylation (1,3) of aromatic compounds (1,1) in the presence of a metallic catalyst (1,2) and the corresponding halide. It is formulated in a general form. (Scheme 1)

The catalysts often used are the Lewis acids and a wide variety of these namely $AlCl_3$, $AlBr_3$, BF_3, $TiCl_4$ have been used. Solvents such as CS_2, CH_2Cl_2, n-hexane, nitrobenzene or CH_3NO_2 are used in excess. Solvent such as diethyl ether which can co-ordinate with these reagents markedly decrease the yield of the products. These relations have been critically reviewed in a series of books edited by Olah [1].

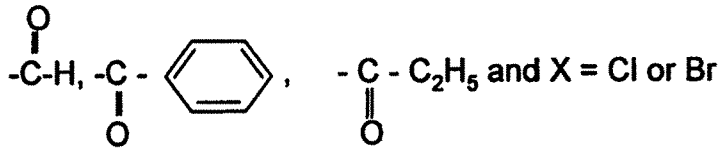
Chart - 1

Scheme - 1

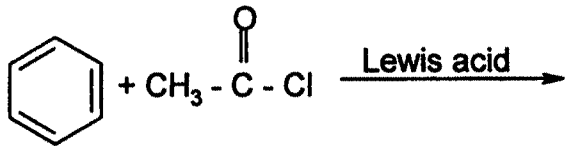


(1) **(2)** **(3)**

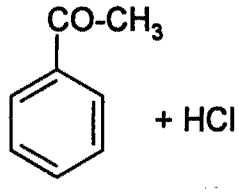
Where R = CH_3, C_2H_5, $CH(CH_3)_2$, $C(CH_3)_3$, $-\overset{O}{\overset{\|}{C}}-CH_3$



Scheme - 2



(1) **(4)**



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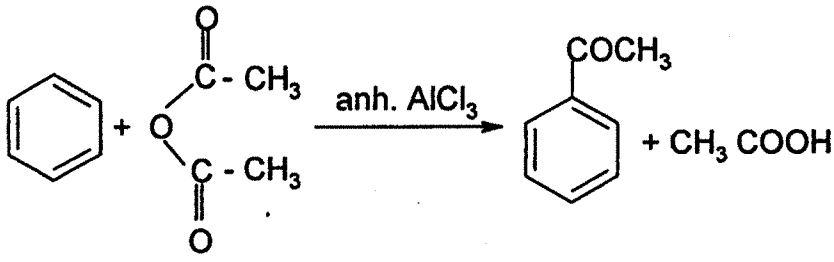
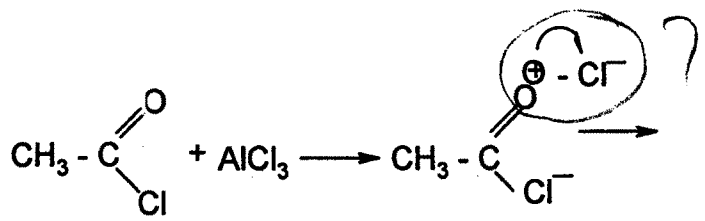


(1) **(6)** **(7)**

CHART - 2

Scheme - 3



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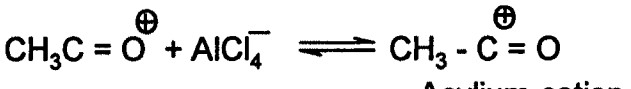
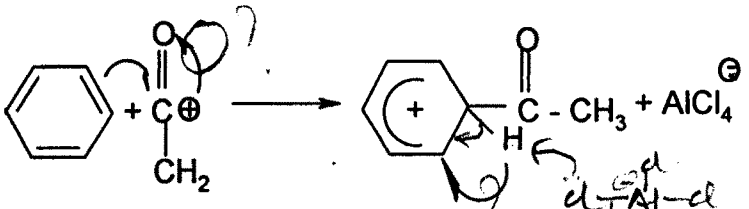
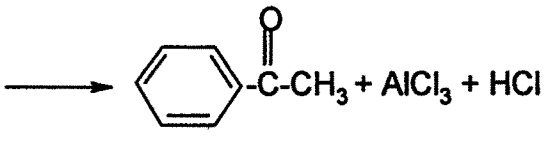


Acylium cation

(8)

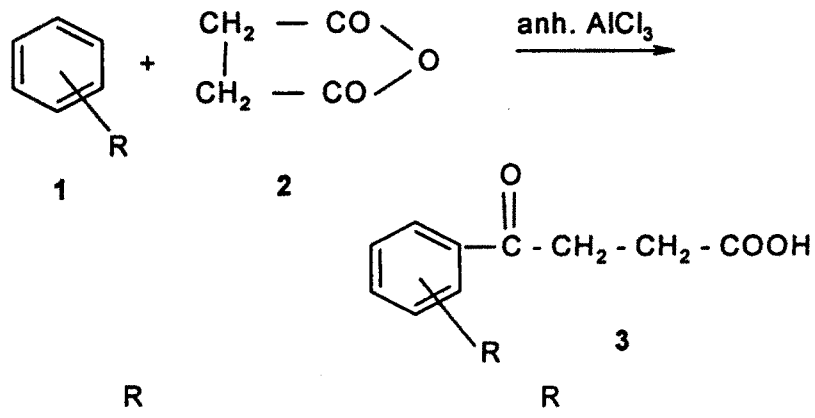


(9) **(10)**



(11)

CHART - 1



R R

1 a) R= H

1 b)R=-CH_3

1 c) R=-OCH_3

3 a) R= H

3 b)R=-CH_3

3 c) R=-OCH_3

Mechanism

1.

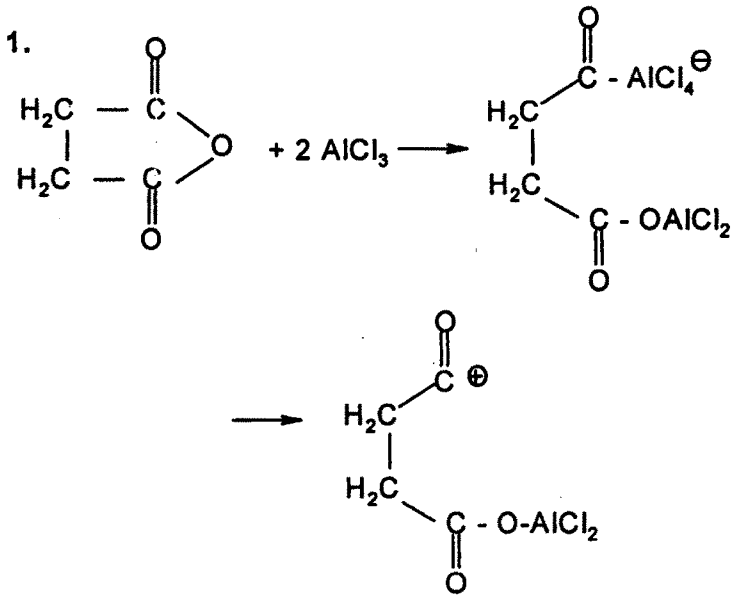
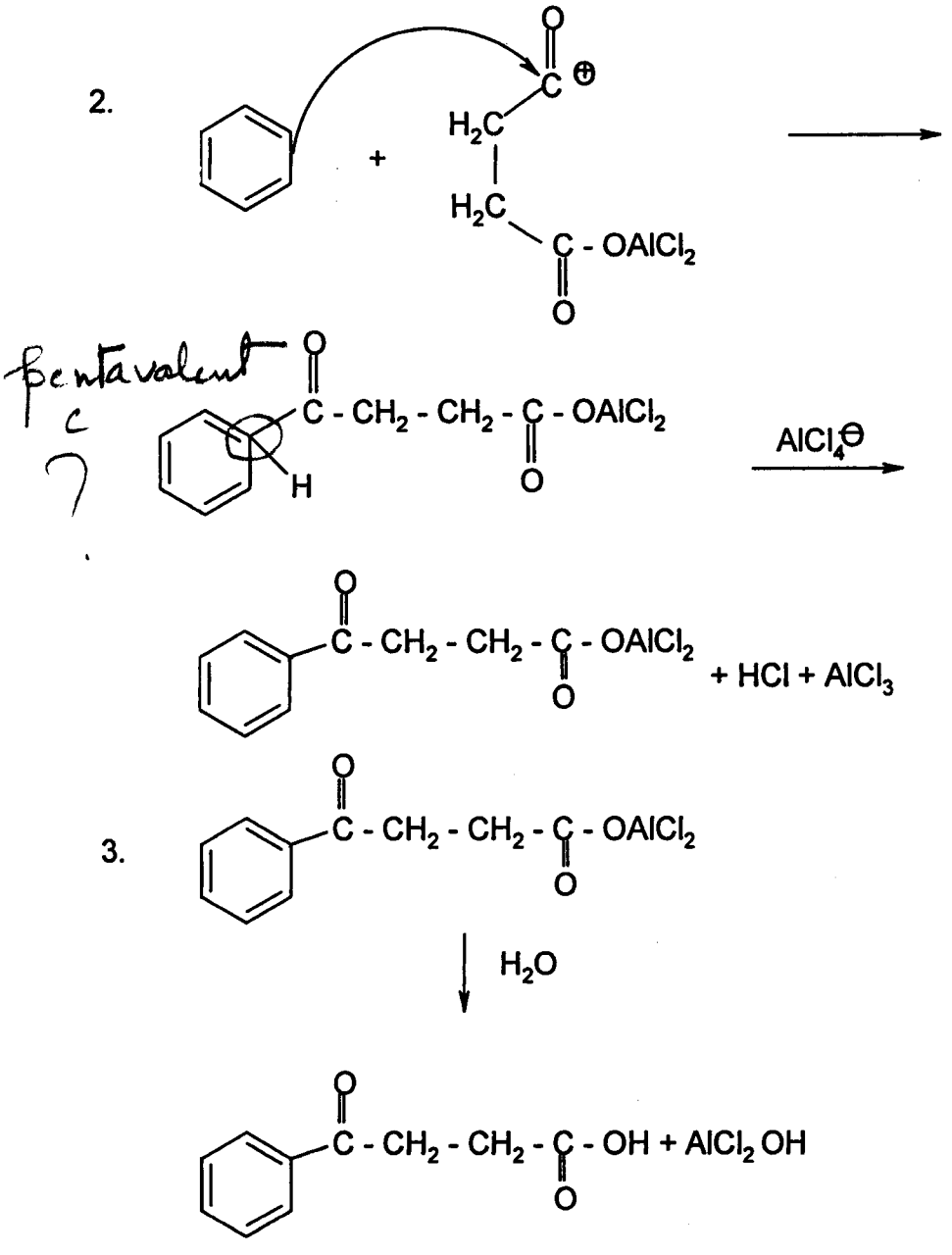


CHART - 2


2.
+
H_2C
H_2C
C - $OAlCl_2$
O
C ⊕
pentavalent
C - CH_2 - CH_2 - C - $OAlCl_2$
H
$AlCl_4$ ⊖
+ HCl + $AlCl_3$
3.
H_2O
C - CH_2 - CH_2 - C - OH + $AlCl_2$ OH

The Friedel-Craft reaction has proven to be of great utility in preparing ketones, ring closure products and anthraquinones[1]. Olefins and alcohols can also be employed in place of alkyl halides with protic acids (H_3SO_4, H_3PO_4,HF, PPA) as catalysts. Among the alkyl halides the reactivity decreases in the order F>Cl>Br>I. The effect of temp and the nature of the catalyst is important for product distribution. Side reactions such as isomerisation of the product and dealkylation i.e. the replacement of an alkyl group by hydrogen are common features of this reaction.

For this observations a possible explanation may be that above reaction is reversible and only thermodynamically stable products are obtained. Acylation in contrast to alkylation is irreversible. This is attributed to the resonance stablilization existing between the acyl group and the aromatic nucleus. But reversibility can be obtained by twisting the acyl group out of the plane of the ring by ortho substituents.

Friedel-Craft acylation is a reaction carried out to introduce an acyl group (1,4) o
||
(-C-R) into an aromatic nucleus. For this, aromatic compound is treated with an acyl halide or an acid anhydride (1,6) in the presence of a Lewis acid like anhydrous aluminium chloride or boron trifluoride. The reaction is exothermic and is carried out in solvents such as carbon disulphide, nitorbenxene, methylene dichloride. The product is an aromatic ketone. (1,5)(1,7)

This is an electrophilic aromatic substitution involving the formation of acylium cation ($R\text{-}Co^+$). The chloride forms a complex with Lewis acid. Then it splits up to give an acylium cation. (2,8)

Then the acylium cation, (2,9) attacks the π electron of the aromatic ring forming a benzenomium ion (2,10) which is stabilized by resonance.

Finally the benzonium ion loses a proton in presence of the anion to from the substituted product. (Scheme 3)

Aromatic rings having activating groups such as alkyl, alkoxy, halogen, acetamide etc are easily acylated. For reasons of steric factors acylation mainly occurs at paraposition. Heterocyclic compounds undergo acylation with good yield. It is mainly used for preparing different aromatic ketones. The acylated product can be converted into alkyl benzenes. The product of acylation can be sometimes cyclized under mild conditions. This can be used in the preparation of higher carbocyclic rings and also some poly nuclear hydrocarbons.

EXPERIMENTAL

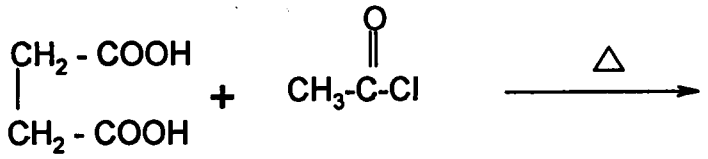
General :

Succinic acid	(s.d. fine – CHEM)
Maleic acid	(s.d. fine – CHEM)
Anhydrous $AlCl_3$	(s.d. fine – CHEM)
Toluene	(s.d. fine – CHEM)
Anisole	(s.d. fine – CHEM)
α-Keto Glutaric acid	(s.d. fine – CHEM)
Acetyl chloride	(s.d. fine – CHEM)
Benzene A.R.	(qualigens)
Conc. H_2SO_4 A.R.	(s.d. fine-CHEM)
Ethyl alcohol	(anhydrous)
Maleic Anhydride	(s.d. fine CHEM)

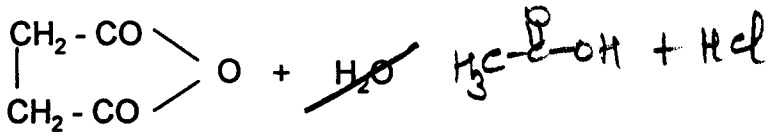
Succinic Anhydride – (1,3)

A mixture of succinic acid (1,1) (59 g) and Acetyl chloride (1,2) (107.5 ml) in a 500 ml round bottom flask was refluxed on water bath (1,2 hours). After some time reaction mixture was allowed to cool in ice bath and solid was

CHART - 1



1 2



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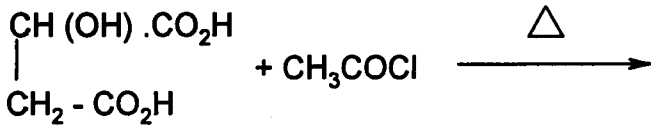

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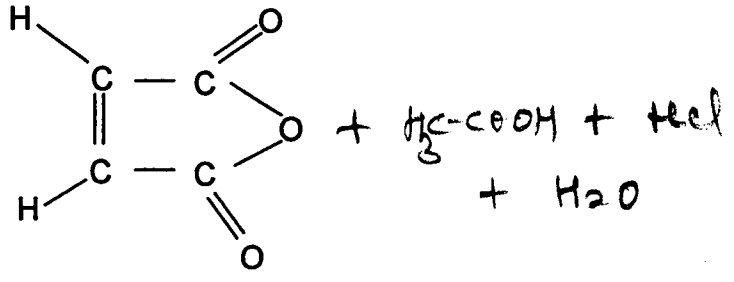

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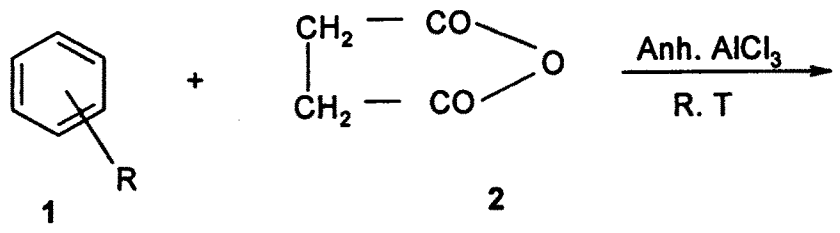
CHART - 2



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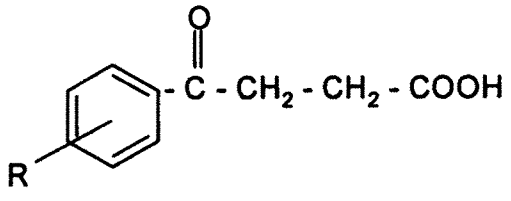


3

R

1 a) R = H

1 b) R = -CH_3

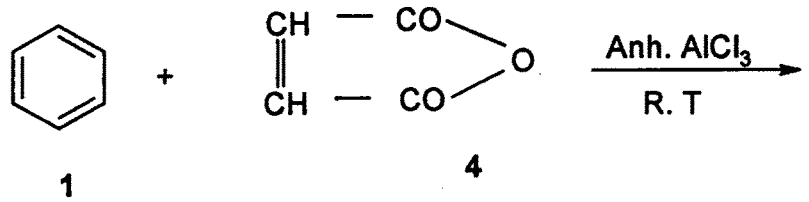
1 c) R = - OCH_3

R

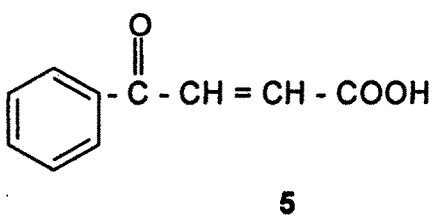
3 a) R = H

3 b) R = -CH_3

3 c) R = - OCH_3



1 4



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separated. The solid compound was filtered and washed with 40 ml of anhydrous ether. It was dried by using vacuum desiccator and product was obtained (40 g) M.P. = 117° – 118°C (Lit. 118-119°).

Maleic Anhydride (1,5)

A mixture of Maleic acid (1,4) (45g, 0.33 mol) and acetyl chloride (1,2) (63g, 57 ml, 0.835 mol) was taken in a 250 ml round bottom flask. Attach a reflux condenser and connect the top of it to a gas absorption trap. The flask was refluxed gently on water bath to start the reaction, when hydrogen chloride is evolved and maleic acid passes into solution. When the evolution of gas ceases, heat the flask on water bath for 1-2 hours.

When a fraction of low boiling point passes over first then temperature rises rapidly to 190°C. Maleic anhydride was collected at 195-200°C. It was further recrystalised from chloroform, we get pure maleic anhydride (18g) M.P. = 52-53°C (lit. 54°C).

1) β-Benzoyl propionic acid (2,3)

A mixture of dry benzene (2,1) (9.5 ml) and succinic anhydride (2,2) (17g) was placed in a 500 ml three necked flask with a mechanical stirrer and two efficient reflux condensers. Powdered anhydrous $AlCl_3$ (50g) was added at a time and the reaction mixture was stirred. While stirring hydrogen chloride was evalued and reaction mixture becomes hot. After cooling the reaction mixture, water (75 ml) was added slowly through separatory funnel. Concentrated hydrochloric acid (25 ml) was added and benzene was removed by steam distillation. When the hot mixture was transferred to beaker (500 ml), the β-Benzoyl propionic acid was separated as a colourless oil and later it solidified. It was kept in ice for cooling and then filtered off. Washed with diluted hydrochloric acid (50 ml) and cold water (50 ml). Crude acid was dissolved in solution of anhydrous sodium carbonate with water and boiled for 10-15 min. Aluminium hydroxide was removed by filtration and washed with hot water. Decolourising carbon (2g) was added to remove the colour and was stirred for 5 min and filtered. Hot filtrate was transferred to beaker, at 50°C it

was acidified with hydrochloric acid. Again it was cooled to 0°C and washed with cold water. Then it was dried for 12 hrs to yield β-benzoyl propionic acid (15g) M.P. = 114°C (lit. – 116°C).

2) 3-Methyl-β-Benzoyl pripionic acid – (2,3b)

A mixture of toluene (2,1b) (40 ml) and succinic anhydride (40g) (2,2) was placed in a 500 ml three necked flask with a mechanical stirrer and two efficient reflux condenser. At once powdered anhydrous $AlCl_3$ (50g) was added and the reaction mixture was stirred. While stirring hydrogen chloride was evalued and reaction mixture becomes hot. After cooling the reaction mixture water (75 ml) was added slowly through separatory funnel. Concentrated hydrochloric acid (25 ml) was added and toluene was removed by distillation. When the hot mixture was transferred to beaker (500 ml) the 3-methyl β-benzoyl propionic acid was separated as a colourless oil and later it solidified. It is kept in ice for cooling and then filtered off, washed with dilute hydrochloric acid (50 ml) Crude acid was dissolved in solution of anhydrous sodium carbonate with water and boiled for 10-15 min. Aluminium hydroxide was removed by filtration and washed with hot water. Decolourising carbon (2g) was added to remove the colour and was stirred for 5 min and filtered. Hot filtrate was transferred to beaker at 50°C it was acidified with hydrochloric acid. Again it was cooled to 0°C and washed with cold water. Then it was dried for 12 hrs to yield 3-methyl-β-Benzoyl propionic acid (13g) M.P. = 108°C.

3) p-Methoxy Benzoyl-2-propionic acid (2,3c) :

A mixture of anisole (35.7g, 0.33 mole in nitrobenzene, 150 ml) (2,1c) and succinic anhydride (2,2) (36g, 0.36 mole) was taken in 500 ml round bottom flask. $AlCl_3$ (94 gm, 0.7 mole) was added at 0°C to a stirred solution/mixture. The mixture was stirred overnight at room temperature. It was decomposed over iced-HCl and stirred for 15 min. Solid which was separated in the reaction mixture was filtered , washed with 3x100 ml pet ether to remove nitrobenzene. The product ~~which was~~ obtained, it was further recrystalised

with ethanol to get pure product of p-methoxy Benzoyl-2-propionic acid (22g) M.P. = 138°C. (lit. 146°).

4) 3-Benzoyl-2-propenoic acid (2,5)

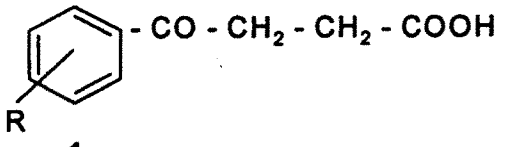
$AlCl_3$ (65g) was added at once in a stirred solution/mixture of benzene (2,5) (115 ml) and maleic anhydride (2,4) (125g) which was taken in a 500 ml three necked flask with a mechanical stirring. 3-Benzoyl-2-propenoic acid was prepared by similar method given as above (17g) M.P. = 58°C.

General procedure for esterification of acids :

A mixture of acid, ethyl alcohol and conc. Sulphuric acid A.R. was taken in a reaction flask fitted with a water condenser and it was refluxed for 8-10 hrs at ~~temperature~~ 60°C. Then reaction mixture was allowed to cool. After removal of solvent under vacuum, the residue was diluted with water. The organic layer was extracted with ether and washed with aqueous sodium bicarbonate, water and dried. Removal of solvent furnished the ethyl ester and it was purified by vacuum distillation.

No.	List of esters	Wt. of Product	MP/BP
1)	Ethyl-β-benzoyl propionate (3,3a)	5 gms	95°C/10mm
2)	Ethyl-β-benzoyl propeonate (3,5)	5 gms	52°C/10mm
3)	α keto diethyl glutarate (3,7)	6 gms	125°C/10mm
4)	Ethyl-3-(-4 methoxy benzoyl)-propionate (3,3b)	7 gms	51°C

CHART - 3

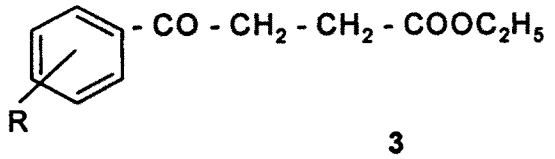


1

+ C_2H_5OH

2

Conc. H_2SO_4



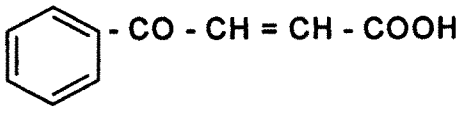
3

1 a) R = H

1 b) R = OCH_3

3 a) R = H

3 b) R = OCH_3



4

+ C_2H_5OH

2

Conc. H_2SO_4

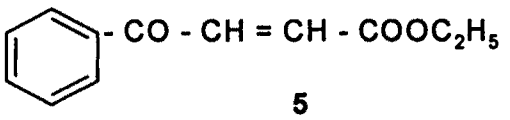


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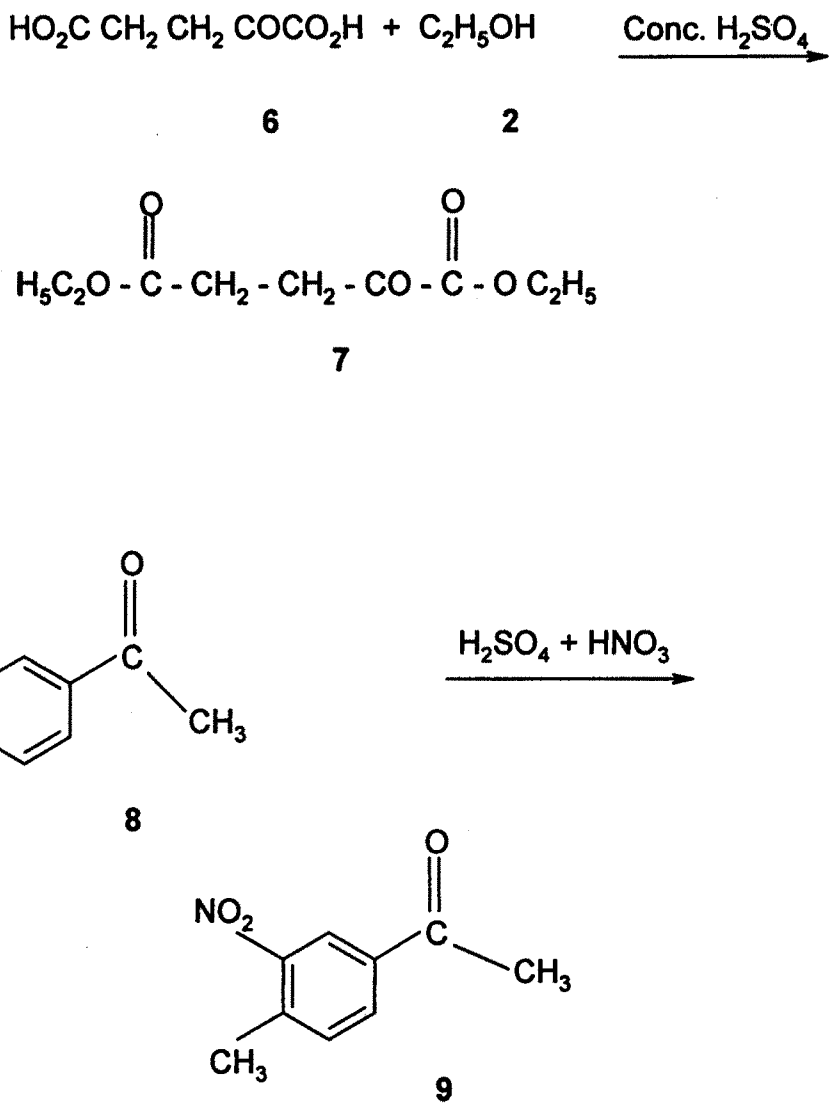
CHART - 4


HO2C CH2 CH2 COCO2H + C2H5OH
Conc. H2SO4
6
2
H5C2O - C - CH2 - CH2 - CO - C - O C2H5
7
H2SO4 + HNO3
CH3
CH3
8
NO2
CH3
CH3
9

PREPARATION OF 3-NITRO-4-METHYL ACETOPHENONE [2]

To a well stirred solution of concentrated sulphuric acid (30 ml) cooled at –20° was added slowly 4-methyl acetophenone (7.5g) (4,8) followed by the cooled solution of nitrating mixture consisting of conc. sulphuric acid (12ml) nitric acid (7.5ml). The reaction mixture was stirred at –15°C for 30 min. more and then poured over crushed ice. Pale yellow solid was obtained (8g) (4,9) after filtration and was washed with water till free from acid. Crude product was purified by crystallization M.P. 60° (ethanol) (lit. M.P. 60-61°).

MATERIALS AND METHODS

MICROORGANISM

The microorganism Rhizopus arrhizus was obtained from National collection of Industrial Microorganism (NCIM), National Chemical Laboratory Pune. The fungus was grown on a modified Czapek-Dox medium.

PROPOGATION OF THE CULTURE

The propogation of culture was done on potato extract, dextrose and agar contained medium. The following compositions were used and pH of the medium was adjusted to 5.8.

Potato Extract	20% w/v
Dextrose	2%
Agar	2.3%
Distilled water	1 lit

The prepared medium was steamed to form a homogeneous melt and aliquots (10ml) were poured into test tubes, then it was autoclaved subsequently at 15 p.s.i. for 20 minutes. These were allowed to solidify after slanting. The slants were inoculated with the fungus spores from the stock culture and

incubated at 28°C for three days when maximum growth was observed. The fungus R. arrhizus was subcultured every 2-3 months.

MEDIUM

The modified Czapek-Dox medium [4] of the following composition had been used in the experiments.

Ferrous sulphate A.R. (Thomas Baker)	30 mg
Dipotassium hydrogen phosphate A.R. (Thomas Baker)	150 mg
Magnesium sulphate A.R. (Thomas Baker)	750 mg
Potassium chloride A.R. (s.d. fine-CHEM Ltd.)	1.50 g
Potassium dihydrogen phosphate A.R. (Thomas Baker)	2.85 g
Sodium Nitrate A.R. (s.d. Fine-Chem Ltd.,)	6 g
Yeast extract (Qualigens)	1.5 g
Corn Steep liquor (Sukjit Chemicals, India)	15 g
Glucose A.R. Thomas Baker	120 g
Distilled water	3 lit
pH adjusted to 4.5 – 4.8	

An equal distribution of medium (150 ml) was done in 500 ml cotton plugged Erlenmeyer flasks.

CONDITIONS FOR INCUBATION

The inoculation of fungus R. Arrhizus was carried out in an autoclave having 500 ml cotton plugged Erlenmeyer flask contained with 150 ml medium (15 p.s.i. for 20 minutes). The flasks were shaked at 28 ± 1°C on a rotatory shaker (Remi Instruments,) at 220 r.p.m. for 72 hours. To obtain maximum optical purity and chemical yield, the substrates (100 mg/ml ethanol) were incubated with R. arrhizus for varying time intervals.

THE ISOLATION OF THE MICROBIAL METABOLITES

General procedure

The mycelial mass was removed at the end of fermentation period by filtration. The filtrate was extracted with chloroform (3 x 10 ml). This extract was washed with water (10 ml) and dried over anhydrous sodium sulphate (2gm). Under reduced pressure the solvent was removed to furnish an oily liquid, this was subjected to preparative TLC to yield the microbial products. The hot acetone was used to destroy mycelial mass. After removing solvent the viscous liquid remained was further diluted with water and extracted with ethyl acetate (3x 10 ml).The organic layer was washed with water (10ml) dried over anhydrous sodium sulphate and the solvent was removed under reduced pressure to yield a gummy residue.

CONTROL EXPERIMENTS

The substrates and R. Arrhizus were incubated for varying time intervals. Isolation of the substrates were almost quantitative (~ 95%) after the work of the fermentation broth.

ORGANISM CONTROL

In this experiments R. Arrhizus was cultivated in Czapek-Dox medium (150 ml) without the substrate for varying time intervals. By using standard procedure work up of the fermentation broth was carried out, which yielded only fungal fatty materials.

THIN–LAYER CHROMATOGRAPHY

TLC plates for qualitative work were prepared (20 x 20 cm) using a slurry containing silica gel GF – 254 (Qualigens Fine Chemicals) and distilled water. Plates were initially dried at room temperature and then activated for one hour at 100°C in oven. Further, plates were developed with suitable solvent system and examined under ultra violet (254 nm) lamp for the detection of spots. The following reagents used for visualization were viz. iodine vapour, alcoholic ferric chloride, sulphuric acid (10%) and 2,4 dinitrophenyl hydrazine.

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