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STUDIES ON SOME α,β-UNSATURATED NITRILES

M.M. Mohamed and M.A. El Hashash

Chemistry Department, Faculty of Science, Ain Shams University, Cairo, Egypt

A.El. Naggar, F. Said and W.M. Ali

Chemistry Department, Faculty of Science, El-Azher University, Cairo, Egypt

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INTRODUCTION

Previously [1] we had reported the behaviour of some α,β-unsaturated arylidene malononitriles and other related compounds toward the action of active methylene compounds and other reactions. However, in this study we extend our investigation upon arylidene malononitriles (I) for the object of their biological evaluation.

The arylidene malononitriles (I) react with active methylene compounds, Grignard reagents, diazomethane and hydrazine hydrate and they gave compounds (II–XIII).

The antimicrobial and antifungal activities of the prepared organic compounds were determined.

$$\underset{\text{I}}{\text{Ar-CH=C(CN)}_2}$$

Where Ar, a = C_6H_5; b = *p*-ClC_6H_4; c= *p*-$(CH_3)_2$ NC_6H_4; d= *p*-$NO_2C_6H_4$; e= 3,4- CH_2O_2 C_6H_3; f = *m*-NO_2 C_6H_4.

When arylidene malononitriles (Ia–c) are treated with cyclohexanone in the presence of sodium methoxide at room temperature, they give the Michael adducts [α-2-oxocyclohexyl) benzyl or *p*-chlorobenzyl or *p*-(dimethylamino)-benzyl] malononitrile (IIa–c).

The reaction is conducted in absolute alcohol, and the nucleophilic addition of carbanions to α,β-unsaturated nitrile compounds (I) result in the formation of carbon–carbon bond.

The IR spectra of II show strong absorption bands at 2240–2220 cm^{-1} attributable for ν C≡N, at 1710–1730 due to ν C=O of cyclic ketones, and at 2940–2920 cm^{-1} due to ν CH.

When the arylidene malononitriles (Ia–c) are allowed to condense with acetylacetone under the Michael reaction conditions, they result in the formation of [α-(1-acetylacetonyl)-benzyl or *p*-chlorobenzyl or *p*-(dimethylamino) benzyl] malononitrile (3a–c). The IR spectra of the products (IIIa–c)revealed strong absorption bands at 1720–1700 attributable for νC=O at 2220–2200 due to νC≡N and at 2900 cm^{-1} due to νCH.

The base-catalyzed Michael addition of ethyl acetoacetate to *p*-chlorobenzylidene malononitrile (Ib) in the presence of piperidine gives ethyl 4,4-dicyano-3-(*p*-chlorophenyl)-2-acetylbutyrate (IV). The IR spectrum of IV shows bands attributable for ν_{max} of two carbonyl groups at 1720–1670, ν C≡N at 2200 and ν CH at 2940–2900 cm^{-1}

The NMR spectrum of compound IV in $CDCl_3$ can be interpreted as follows:

A complex pattern at δ 7.3 p.p.m. represents the aromatic protons, a triplet of the methyl group CH_3–(splitting by the -CH_2–hydrogens) at δ 1.15 p.p.m., a quartet at δ 4.1 p.p.m. represents the methylene ester –CH_2 (splitting by the CH_3–hydrogens), a signlet at δ 2.39 p.p.m. represents CH_3–CO (ketone), and finally all –CH's appear at δ 4.51 p.p.m. as a multiplet.

Similarly, ethyl phenylacetate reacts with *p*-dimethylaminobenzylidene malononitrile (Ic) under reflux and yields ethyl 4,4,-dicyano-3-[*p*-(dimethylamino) phenyl]-2-phenylbutyrate (V). The IR spectrum of V shows absorption bands attributable for ν C=O of ester at 1720, ν C≡N at 2200 and ν CH at 2900 cm^{-1}

Cyanoacetic acid adds to the arylidene malononitriles (Ib and Ic) under the Michael reaction conditions and gave the Michael adducts α-cyano-β-(dicyanomethyl)-*p*-(chloro or dimethylamino) hydrocinnamic acids (VIa and VIb) respectively.

The assigned structure for the products (VIa and VIb) is infered from the following: (i) Compounds (VIa and VIb) are easily soluble in sodium carbonate solution. (ii) The IR spectra of compounds (VIa and VIb) show strong absorption bands at 1700–1690 due to ν C=O of acid, at 2240–2200 due to ν_{max} of two C≡N groups, at 2960–2860 due to ν CH and at 3390–3200 cm^{-1} due to ν OH stretching vibrations.

By analogy with the recently reported reaction of arylidene malonic ester with nitromethane [2], compound (Ic) adds nitromethane in the presence of sodium ethoxide at room temperature to give the Michael adduct [*p*-(dime-

thylamino)-α-(nitromethyl) benzyl] malononitrile (VII). The IR spectrum of VII shows bands attributable for ν -C-NO_2 at 1550, ν C≡N at 2200 and ν CH at 2890 cm^{-1}

It has been reported that [3], diethyl phosphorous acid add to the α,β--double bond in ethylidene malonic ester in the presence of RONa/ROH mixture on a steam-bath.

In this study, we have found that, compounds (Ib and Ic) add dimethyl phosphite in the presence of CH_3OH/ CH_3ONa mixture and gave dimethyl [*p*-(chloro or dimethylamino)-α-carbamoyl-α-cyanomethylbenzyl] phosphonate(VIIIa and VIIIb). The structure of compounds (VIII) was proved from their IR spectra which reveals the presnece of ν P=O at 1290, ν C≡N at 2200, ν CH at 2860, ν $CONH_2$ at 1660–1640 and ν NH at 3360–3280 cm^{-1}

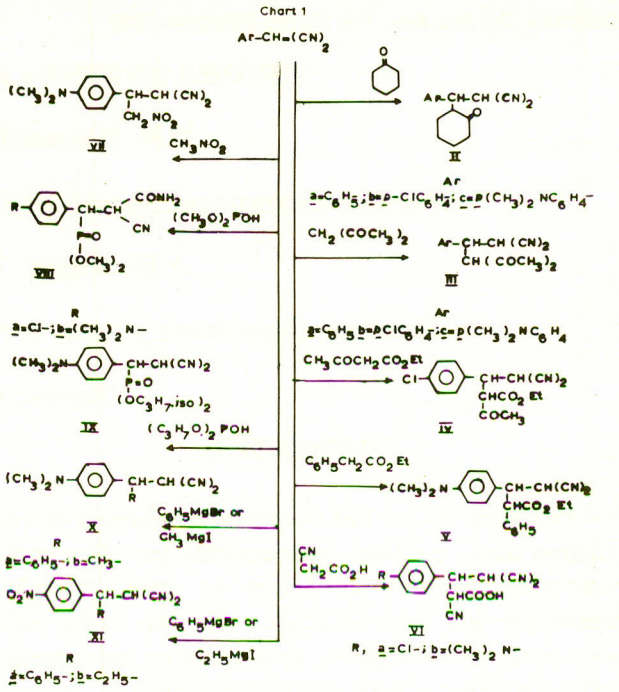
Similarly, compound (Ic) adds diisopropyl phosphite in the presence of isopropanol and sodium isopropoxide yielding diisopropyl α-(dicyanomethyl)-*p*-(dimethylamino)-benzyl-phosphonate (IX). The IR spectrum of IX shows well-defined absorption bands attributable for ν P=O at 1300, ν C≡N at 2200 and ν CH at 2860 cm^{-1}.

It seemed of interest to study the behaviour of α,β-unsaturated nitrile system in arylidene malononitriles (I) towards the action of Grignard reagents.

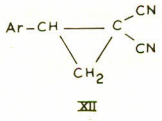
We have found that, *p*-dimethylaminobenzylidene malononitrile (Ic) reacts with phenyl magnesium bromide or methyl magnesium iodide in ethereal solution to yield [*p*-(dimethylamino)-α-phenyl or methylbenzyl] malononitrile (Xa and Xb).

Similarly, *p*-nitrobenzylidene malononitrile (Id) reacts under the same reaction conditions with phenylmagnesium bromide and ethylmagnesium iodide to give (*p*-nitro-α-phenyl or ethylbenzyl) malononitrile (XIa and XIb) respectively. The IR spectral data of compounds (X and XI) show strong absorption bands at 2220–2200 due to ν C≡N and at 2940–2850 cm^{-1} due to ν CH. The NMR spectrum of compound Xa in $CDCl_3$ show signals at δ 1.1 p.p.m. (d) corresponding to the methine proton (Ar–C*H*–), at δ 3.1 p.p.m. (s) corresponding to six protons of $-N(CH_3)_2$ group, at δ 4.4 p.p.m. (q) corresponding to the other methine proton $-CH(CN)_2$, at δ 6.8 p.p.m. (d), at δ 7.3 p.p.m. (d) two doublets corresponding to four aromatic protons of the substituted-phenyl group $(CH_3)_2NC_6H_4-$ and finally at δ 7.5 p.p.m. (m) corresponding to five aromatic protons of the phenyl group. All the above reactions are represented in Chart 1.

Recently, it has been reported that, treatment of 5-arylidene derivatives of barbituric acid with ethereal diazomethane lead to the formation of a colourless cyclopropane compounds [4]. In this study, the treatment of arylidene malononitriles (Ib–e) with equimolar amounts of diazo-



methane in cold dry diethylether gave 2-[*p*-(chloro- or dimethylamino- or nitro)-phenyl or (3,4-piperonyl)]-1,1-cyclopropanedicarbonitrile (XII a–d).



a = *p*- ClC_6H_4-; b= *p*-$(CH_3)_2$ NC_6H_4-; c= *p*-NO_2 C_6H_4-; d= 3,4-CH_2O C_6H_3-.

In support for the structure assignment for the cyclopropane derivatives (XIIa–d) are the following: (i) Correct analytical values. (ii) The IR spectral data of compounds (XIIa–d) reveals the presence of cyclopropane ring system absorbs at 1020–1000, ν C≡N at 2220–2200 and ν CH at 2960–2890 cm^{-1}.

The reactivitiy of the olefinic double bond conjugated with the cyano-group has been receiving increasing interest in the reaction with hydrazine hydrate.

Few examples concerning the reaction between arylidene malonic ester and hydrazines have been recorded in the literature [5,6].

In the present work, arylidene malononitriles (Ia–d and f) react with hydrazine hydrate in ethanol at room temperature (20^o) to give the corresponding azines (XIIIa–e) and malononitrile.

$$Ar - CH = N-N=CH-Ar$$

XIII

a= C_6H_5-; b= *p*- ClC_6H_4-; c= *p* $(CH_3)_2N$ C_6H_4-; d= *p*-NO_2 C_6H_4-; e= *m*-NO_2 C_6H_4-.

Table 1. Biological activities of the synthesized organic compounds.

Compound No.	*Bacillus subtilis*	*Bacillus cereus*	*Bacillus mycoides*
Ia	–	–	–
Ib	+(500)	+(500)	–
Ic	++(250)	–	–
Id	–	–	–
Ie	–	–	–
If	+++(31)	++(250)	+++(60)
IIa	–	–	–
IIb	+(500)	–	+(500)
IIc	–	–	–
IIIa	–	–	–
IIIb	–	–	–
IIIc	–	–	–
IV	–	–	–
V	–	–	–
VIb	–	+(500)	–
VII	–	–	–
VIIIa	–	–	–
Xa	–	–	–
Xb	–	–	–
XIa	++(250)	+(500)	+(500)
XIb	++(250)	+(500)	–
XIIa	++(250)	+(500)	++(250)
XIIb	–	–	–
XIIIa	–	–	–
XIIIb	–	–	–
XIIIc	–	–	–
XIIId	++(250)	–	–
XIIIe	–	–	–

The IR spectra of compounds XIII revealed the presence of ν C=N at 1660–1640 and ν CH at 2940–2860 cm^{-1}

Biological Activity of the Synthesized Organic Compounds. The biological activities of the synthesized organic compounds (*cf.* Table 1) were tested against different types of microorganisms and fungi, e.g. *Bacillus subtilis, Bacillus cereus, Bacillus mycoides, Escherichia coli, Salmonella typhos* and *Penicelium.*

We have found that, all the synthesized organic compounds were completely inactive against the three types *Escherichia coli, Salmonella typhosa* and *Penicelium.* However, *p*-chlorobenzylidene malononitrile (Ib) was found to be highly active against *Bacillus subtilis* and *Bacillus cereus* and inactive against *Bacillus mycoides. p*-Dimethylaminobenzylidene malononitrile (Ic) was found to be highly active against *B. subtilis* but inactive against *B. cereus* and *B. mycoides.* On the other hand, *m*-nitrobenzylidene malononitrile (If) was found to be highly active against the three microorganisms *B. subtilis, B. cereus* and *B. mycoides.* However, the remaining arylidene malononitriles were inactive towards the tested microorganisms. Similarly, [*p*-chloro-α-(2-oxocyclohexyl)benzyl] malononitrile (IIb) was found to be highly active against *B. subtilis* and *B. mycoides* but inactive against *B. cereus.* In contrast with compound IIb, compound VIb was found to be highly active against *B. cereus* and inactive against the other two types *B. subtilis* and *B. mycoides.* Also, compounds (XIa and XIIa) were found to be highly active against the three types *B. subtilis, B. cereus* and *B. mycoides.*

Incorporation of ethyl- group in compound XIb instead of phenyl group in compound XIa decrease the biological activity of compound XIb and it was found to be inactive against *B. mycoides* but it was highly active against the other two types *B. subtilis* and *B. cereus.*

The azine derivative (XIIId) was the only compound in the azine series (XIII) which has activity against *B. subtilis* only.

Table 2. Structure and reactivity of the compounds.

Compounds	Structure	*B. subtilis*	*B. cereus*	*B. mycoides*
Ib	*p*-Chlorobenzylidene malonitrile	+(500)	+(500)	–
Ic	*p*-Dimethylaminobenzylidene malonitrile	++(250)	–	–
If	*m*-Nitrobenzylidene malonitrile	+++(31)	++(250)	+++(60)
VI	[α-2-Oxocyclohexyl-*p*-chlorobenzyl] malonitrile	+(500)	–	+(500)
VIb	α-Cyano-β-(dicyanomethyl)-*p*-(dimethylamino) hydrocinnamic acid	–	+(500)	–
XIa	(*p*-Nitro-α-phenylbenzyl) malonitrile	++(250)	+(500)	+(500)
XIb	(*p*-Nitro-α-ethylbenzyl) malonitrile	++(250)	+(500)	–
XIIa	2-[*p*-(Chloro-) phenyl]-1,1-cyclopropane-dicarbonitrile	++(250)	+(500)	++(250)
XIIId	*p*-Nitrobenzylideneazine	++(250)	–	–

Table 3. Michael adducts II and III.

Compound	M.p. colour	Solvent yield (%)	Formula (M.wt.)	Analysis (%) Required	Found
IIa	115 Orange	LP/Bz 47	$C_{16}H_{16}N_2O$ (252)	C 76.19 H 6.35 N 11.11	76.42 6.44 11.32
IIb	100 Pale brown	LP/Bz 36	$C_{16}H_{15}N_2ClO$ (286.5)	C 67.01 H 5.24 N 9.77	67.23 5.32 9.92
IIc	170 Brown	LP/Bz 52	$C_{18}H_{21}N_3O$ (295)	C 73.22 H 7.11 N 14.23	73.32 7.24 14.52
IIIa	140 Yellow	LP/Bz 41	$C_{15}H_{14}N_2O_2$ (254)	C 70.86 H 5.51 N 11.02	71.08 5.43 11.20
IIIb	145 Pale yellow	LP/Bz 34	$C_{15}H_{13}N_2ClO_2$ (292.5)	C 61.53 H 4.44 N 10.94	61.67 4.18 11.22
IIIc	165 Orange red	LP/Bz 44	$C_{17}H_{19}N_3O_2$ (297)	C 68.68 H 6.39 N 14.14	68.53 6.13 14.00

LP, light petroleum (b.p. 40–60°); Bz, benzene.

The high biological activity of compounds (Ib, If, IIb, XIa, XIb and XIIa) may be attributed to the presence of chloro- or nitro groups in their structures. On the other hand, compounds (Ic and VIb) which contain the *p*-dimethylamino group become of less activity (cf. Table 2).

The remaining organic compounds were found to be biologically inactive towards all the tested microorganisms.

The important results of the high biological properties of the synthesized organic compounds revealed that, the substitution of chloro- or nitro- group in their structure may create an interesting biological activity for these organic compounds, however, the unsubstituted chloro- or nitro- groups were found to be of very low biological properties.

EXPERIMENTAL

The IR absorption spectra were determined with a Unicam SP 200G spectrophotometer using KBr wafer technique. The NMR spectra were measured with Varian VN 1009 (S-60T). M. ps. are uncorrected.

Base-Catalyzed Michael Addition of Cyclohexanone or Acetylacetone to Arylidene Malononitriles (Ia-c). Formation of the Malononitrile Derivatives IIa–c and IIIa–c. A mixture of arylidene malononitriles (Ia–c) (0.02 mole) and cyclohexanone or acetylacetone (0.02 mole) in sodium methoxide (0.02 mole) was set aside at room temperature for four days. The reaction mixture was poured into crushed ice, extracted with ether to get rid of any unreacted organic materials. The cooled aqueous layer was acidified with cold dil HCl and the obtaining precipitate that separated was crystallized from the suitable solvent to give (IIa–c) and (IIIa–c). The results are listed on Table 3.

Base-Catalyzed Michael Addition of Ethyl Acetoacetate to Ib; Ethyl Phenylacetate to Ic; and Cyanoacetic Acid to Ib and Ic). Formation of IV, V and VIa and VIb. A mixture of equimolar quantity of Ib with ethyl acetoacetate; Ic with ethyl phenylacetate or (Ib and Ic) with cyanoacetic acid (0.01 mole: 0.01 mole) in 50 ml ethanol and few drops of piperidine (5–7 drops) was added. The reaction mixture was refluxed for 6 hr then poured into crushed ice. The precipitate that formed was filtered off, washed with water and then crystallized from the suitable solvent to give IV, V and (VIa and VIb). The results are listed on Table 4.

Michael Addition of Nitromethane to Ic. Formation of VII: A mixture of Ic (0.02 mole) and nitromethane (0.02 mole) in sodium ethoxide (0.02 mole) was set aside at room temperature for four days. The reaction mixture was poured into crushed ice, extracted with ether to get

Table 4. Michael adducts IV, V and (VIa and VIb).

Compound	M.p. colour	Solvent yield (%)	Formula (M.wt.)	Analysis (%) Required	Found
IV	165–67 Pale yellow	Ethanol 63	$C_{16}H_{15}N_2ClO_3$ (318.5)	C 60.28 H 4.70 N 8.79	60.40 4.81 8.91
V	192–193 Pale yellow	LP/Bz 25	$C_{22}H_{23}N_3O_2$ (361)	C 73.13 H 6.37 N 11.63	73.20 6.41 11.81
VIa	129–30 Yellow	Ethanol 27	$C_{13}H_8N_3ClO_2$ (273.5)	C 57.03 H 2.92 N 15.35	57.20 3.10 15.50
VIb	205–206 Yellow	LP/Bz 32	$C_{15}H_{14}N_4O_2$ (282)	C 63.82 H 4.96 N 19.85	64.01 5.10 19.97

Table 5. Phosphonate derivatives VIII and IX.

Compound	M.p. colour	Solvent yield (%)	Formula (M.wt.)	Analysis (%) Required	Found
VIIIa	179–80 Yellow	Ethanol 31	$C_{12}H_{14}ClN_2O_4P$ (316.5)	C 45.49 H 4.42 N 8.84	45.50 4.50 8.95
VIIIb	246–47 Pale yellow	Ethanol 29	$C_{14}N_{20}N_3O_3P$ (309)	C 54.36 H 6.47 N 13.59	54.35 6.56 13.62
IX	163 Orangeyellow	Ethanol 27	$C_{18}H_{26}N_3O_3P$ (363)	C 59.50 H 7.16 N 11.57	59.51 7.30 11.84

rid of any unreacted organic materials. The cooled aqueous layer was acidified with cold dil HCl and the precipitate obtained was crystallized from ethanol to give VII as blue-black crystals, m.p. 240–42°, yield 60%. ($C_{13}H_{14}N_4O_2$ (258) requires: C 60.46, H 5.42, N 21.70. Found: C 60.58, H 5.54, N 21.75%).

Base-Catalyzed Michael Addition of Dimethylphosphite to Ib and Ic and Diisopropylphosphite to Ic. Formation of VIIIa and VIIIb or IX. A solution of Ib or Ic(0.01) mole), dimethylphosphite (0.02 mole) (or diisopropylphosphite in case of Ic) in 50 ml absolute alcohol, was treated with a saturated solution of sodium alkoxide (sodium methoxide or sodium isopropoxide) in methanol or isopropanol(20 ml).. The temperature of the reaction mixture was maintained at 20–25°. Stirring was continued for an additional 20 hr at room temperature. The whole reaction mixture was then added to water in which a solid substance was obtained. The solid obtained was crystallized from ethanol to give the Michael adducts VIII or IX. The results are listed on Table 5.

Action of Grignard Reagents on Arylidene Malononitriles (Ic and Id). Formation of X and XI. A solution of Ic or Id (0.01 mole) in dry ether (100 ml) was added to an ethereal solution of phenyl magnesium bromide, methyl or ehtyl magnesium iodide (0.03 mole) in a course of 30 min. The reaction mixture was heated under reflux for 4 hr, left overnight at room temperature and then decomposed with amonium chloride solution. The reaction mixture was shaken with ether to get rid of the organic substance. The ether layer was dried and then set aside at room temperature. After evaporation of the excess ether the

Table 6. The porudcts of Grignard reagents with Ic and Id.

Compound	M.p. colour	Solvent yield (%)	Formula (M.wt.)	Analysis (%) Required	Found
Xa	68 Yellow	LP (b.p. 60–80)/Bz 56	$C_{18}H_{17}N_3$ (275)	C 78.54 H 6.18 N 15.27	78.68 6.32 15.36
Xb	46 Orange	LP (b.p. 40–60) 39	$C_{13}H_{15}N_3$ (213)	C 73.23 H 7.04 N 19.71	73.40 7.61 19.86
XIa	102 Brown	LP (b.p. 40–60)/Bz 46	$C_{16}H_{11}N_3O_2$ (277)	C 69.31 H 3.97 H 15.16	69.52 4.10 15.00
XIb	126 Brown	LP (b.p. 40–60) 54	$C_{12}H_{11}N_3O_2$ (229)	C 62.88 H 4.80 N 18.34	63.01 5.00 18.42

Table 7. The products of diazomethane with Ib–e.

Compound	M.p. colour	Solvent yield (%)	Formula (M.wt.)	Analysis (%) Required	Found
XIIa	212 Pale green	LP/Bz 29	$C_{11}H_7ClN_2$ (202.5)	C 65.18 H 3.45 N 13.82	65.32 3.52 14.01
XIIb	108 Orange	LP/Bz 38	$C_{13}H_{13}N_3$ (211)	C 73.93 H 6.16 N 19.90	74.10 6.20 20.00
XIIc	279 Canary-yellow	Ethanoll 27	$C_{11}H_7N_3O_2$ (213)	C 61.97 H 3.28 N 19.71	62.02 3.30 19.75
XIId	113 Yellow	LP/Bz 25	$C_{12}H_8N_2O_2$ (212)	C 67.92 H 3.77 N 13.20	68.06 3.86 13.32

viscous oil that obtained was triturated with light petrol and the solid products were crystallized from the proper solvent to give X and XI. The results are listed on Table 6.

Reaction of Diazomethane with Arylidene Malononitriles (Ib–e). Formation of (XIIa–d): An ethereal solution of diazomethane (0.01 mole) was added to a suspension solution of arylidene malononitrile (Ib–e) (0.01 mole) in dry ether (30 ml). The reaction mixture was shaken for ½ hr and then set aside at room temperature for further 24 hr. The precipitated material was collected by filtration and then crystallized from the proper solvent to give the cyclopropane derivatives XII. The results are listed in Table 7.

Reaction of Arylidene Malononitriles (Ia–d) and (If) with Hydrazine Hydrate. Formation of Azines (XIIIa–e): A solution of each of the arylidene malononitriles (Ia–d or If) (0.01 mole) in ethanol (50 mol) was treated with hydrazine hydrate (2 ml; 85%) and the solution was left aside at room temperature (20–25°) for 1 hr. The solid that separated was filtered off and crystallized from the suitable solvent to give the corresponding azines (XIIIa–e). The results are listed in Table 8.

Table 8. Azine derivatives XIII.

Compound	M.p. colour	Solvent yield (%)	Formula (M.wt.)	Analysis (%) Required	Found
XIIIa	93 Pale yellow	Ethanol 75	$C_{14}H_{12}N_2$ (208)	C 80.74 H 5.81 N 13.45	80.80 5.93 13.58
XIIIb	192–93 Pale yellow	Ethanol 69	$C_{14}H_{10}N_2Cl_2$ (277)	C 60.64 H 3.61 N 10.10	60.72 3.80 10.25
XIIIc	253 Yellow	Benzene 55	$C_{18}H_{22}N_4$ (294)	C 73.43 H 7.53 N 19.03	73.51 7.62 19.56
XIIId	123 Yellow	LP/Bz 46	$C_{14}H_{10}N_4O_4$ (298)	C 56.37 H 3.35 N 18.79	56.41 3.42 18.84
XIIIe	186–88 Pale yellow	Ethanol 61	$C_{14}H_{10}N_4O_4$ (298)	C 56.37 H 3.35 N 18.79	56.50 3.55 18.87

Antimicrobial and Antifungal Activities of the Prepared Compounds

Sensitivity of Microorganisms to Antimicrobial Compouns. In testing antimicrobial activity of compounds we used more than one test organism to increase the chance of detecting antibiotic principals in the test materials. One filamentous strain of fungi was used to detect the antifungal activity. The sensitivity of a microorganism to antibiotics and other antimicrobial agents can be determined by either the hole-plate method or by the filter paper disc method.

(a) The Hole-plate Method: Maintenance of the Test Organisms: The bacterial cultures were preserved on slants of nutrient agar medium (g% peptone, 0.5; beef extract, 0.15; yeast extract, 0.15; glucose, 0.1; NaCl, 0.5; agar, 1.5 and 100 ml tap water).

The pH was adjusted to 7.0. The slants were prepared by transferring 5 ml of the mixed nutrient agar medium in test tubes, plugged by cotton, sterilized and inoculated after solidification by the test organism. The inoculated slants were incubated for 24 hr at 37° and subsequently they were ready for use.

Solutions to be tested were placed in small depressions in the surface of the medium which has the same composition mentioned before. The semisolid media were inoculated with the test organism and 20 ml were transferred to each petri-dish under asceptic conditions and left to solidify. The cavities were made by cutting out small circules of agar with a sterile 8 mm cork borer from the seeded agar and the agar discs were removed from the plate by a sterile needle creating a hole or a well in the agar. In this particular case, each hole in the agar recieved nearly 0.1 ml of the test solution.

The technique was also used with aqueous solutions by Carlson, Douglas and Bissell [7]. The tested solutions were prepared by dissolving 5 mg of the solid compound in 1 ml ethanol or acetone and 9 ml of distilled water were added to each sample. From each sample 0.1 ml were transferred immediately to each hole in the plate seeded by the specific test organism.

In the case of quantitative or semiquantitative estimation of the minimum inhibitory concentration (M.I.C.) for each sample, 5 mg were dissolved in 1 ml acetone or ethanol and 9 ml water were added. A serial dilution was made from the first tube by transferring 5 ml to a tube containing 5 ml distilled water and mixed thoroughly. From the last tube a similar series of dilutions were made. From each dilution 0.1 ml was transferred to the wells in the seeded plates. The plates were left for 2 hr in a refrigerator to permit diffusion of the tested smaple before growth of the test organism. The plates were transferred and incubated at 30° for 24 hr in the case of bacteria and at 24° for 5 days in case of fungi.

(b) The Filter Paper Disc Method: Small discs of filter paper may be used as containers for the antimicrobial solutions to be assayed or tested.

Vincent and Vincent [8] and Epstein *et al.* [9] dipped

the sterile disc into the solution to be tested placing it on the seeded plate. Paper disc must be of uniform thickness and size, and containing graded amounts of the agent to be tested (or the same amount of different agents if comparison is desired).

To carry out the sensitivity test 5 mg of the sample dissolved in 1 ml aceton and 0.1 ml of the solution are absorbed by each disc, dried and placed on the seeded plate. In case of quantitative or semiquantitative estimation of the M.I.C. by the use of the paper-disc method, 5 mg of the compound dissolved in 10 ml acetone and serial dilutions were made using acetone for dilution. From each dilution 0.1 ml was transferred for each disc, dried and placed on the seeded plate, left for diffusion and incubated at 30° for 24 hr for bacteria and at 24° for 5 days for fungi. The agent diffuse into the agar and prevents growth of the bacterium in a zone around the disc.

Filamentous Mold Fungi as Test Organism. In case of search for antifungal agents, the filamentous fungus *Penicillium chrysogenum* was used as a test organism the following were carried out [10]

A suspension of spores of the fungus was filtered through a thin layer of sterile cotton wool to remove fragments of mycelium. Five ml of the spore suspension were added to 40 ml of Czapek-sucrose agar (g %: sucrose, 2.0; $NaNO_3$, 0.2; KH_2PO_4, 0.1; KCl, 0.05; $MgSO_4.7H_2O$, 0.05; agar, 2.0 and tap water 100 ml) at 45° and mixed.

Sterile petri-dishes each containing 20 ml of solidified Czapek-sucrose agar were warmed to about 45° and flooded evenly with the agar spore suspension (3.5 ml to each dish), the plates were then allowed to harden. The results are recorded in Tables 1 and 2.

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