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Synthesis and Evaluation of Antimicrobial, Cytotoxic, and Genotoxic activities of Substituted Cycloalkanepyrazoles

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Abstract:

A number of 1-substituted-3-methyl-4, 5, 6, 7-tetrahydrocyclohexapyrazoles derivatives, and 1-substituted-3-methyl-4, 5, 6, 7-tetrahydrocyclopentapyrazoles derivatives were synthesized and characterized. These compounds were evaluated for their in vitro antimicrobial, cytotoxic, and mutagenic activities. The results of the biological activities showed that both compound 3c and 3d exhibited antibacterial and antifungal activities. They showed activity against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*, whereas compound 3d was active against *Staphylococcus aureus*, *Enterococcus faecalis*. They also showed activity against *Candida albicans*, *Zygosaccharomyces rouxii*, and *Aspergillus niger*. Compound 3c was the most active as antifungal agent. None of the tested compounds found to have mutagenic or cytotoxic action.

Key words: Substituted cycloalkanepyrazoles, synthesis, antimicrobial, cytotoxicity, mutagenicity

Introduction

Synthetic routes leading to pyrazoles have been extensively studied (Elguero, 1984). This has been stimulated by the biological activities associated with the pyrazole nucleus, and the promising application of such compounds in medicine including their antimicrobial and antifungal effects (Goddard *et al.*, 1977; Secor *et al.*, 1971; Tsurami *et al.*, 1976).

Despite intensive research on the synthesis of substituted pyrazoles, little is known about fused ring pyrazoles (Kashima *et al.*, 2001). In the present work some cycloalkanepyrazoles derivatives were synthesized and evaluated for their biological activities as potential antibacterial and antifungal agents.

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On the other hand, Ames test is well established method for examining mutagenicicty of chemical compounds. This is because of its ability of prescreening for any mutagenicicty and any suspected carcinogenic effects. In addition the test makes possible to distinguish between frameshift and base-pair substitution mutagens (McCann and Ames, 1976).

Materials and Methods:

The starting material acid hydrazides were prepared according to procedure described in literature (Clarke and Davis, 1941). The reactions of acid hydrazides (1a-d) with 2-acetylcyclo-hexanone gave 1-substituted-3-methyl-4,5,6,7-tetrahydrocyclo-hexanpyrazoles (2a-d), as shown in scheme 1.

Preparation of 1-substituted-3-methyl-4,5,6,7-tetrahydrocyclohexapyrazoles, (2a-d):

To a solution of acid hydrazide (1a-d) (10mmol) in absolute ethanol (30ml), 2-acetylcyclohexanone (10mmol) was added. A few drops of concentrated HCl were added as a catalyst. The reaction mixture was stirred under reflux for about one hour, after which time the reaction mixture was cooled to give a precipitate. The precipitate was filtered and re-crystallized, using chloroform/ether. Colorless powder was obtained in all cases.

Preparation of 1-Substituted-3-Methyl-4,5,6-Trihydrocyclopentapyrazoles, (3a-d):

To a mixture of acid hydrazides (1a-d) (10 mmol) in absolute ethanol (30 ml) and 2-acetylcyclopentanone (10 mmol) was added. After the addition of a few drops of concentrated HCl, the reaction mixture was stirred under reflux for 3 hours. When the mixture was cooled down, a colorless precipitate had formed. The solid was filtered and re-crystallized from chloroform/ether.

Melting points were determined on electrothermal-digital melting point apparatus, and were uncorrected. Elemental analysis was determined at M.H.W laboratories, Phoenix, Arizona, USA.

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were determined using a Brucker WB 80-SY, WM-250 and AC-250 spectrometers in deuterated chloroform ($CDCl_3$) with tetramethylsilane (TMS) as an internal standard. Carbon-13-nuclear magnetic resonance (^{13}C-NMR) spectra were recorded on a Brucker WM-250, AC-250 spectrometers. Infrared spectra (IR) were recorded on Perkin Elmer FT-IR SP-2000 spectrometer as potassium bromide (KBr) pellets. Mass spectra were determined on a sector field double focusing unit VG7070 mass spectrometer. Chemicals were purchased from Aldrich and Fluka and were used without further purification.

Bacterial and fungal strains:

For antibacterial activity two Gram-positive microorganisms (*Staphylococcus aureus* ATCC 29213), (*Enterococcus faecalis* ATCC 29212) and two Gram-negative microorganisms (*Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853), were used. In antifungal studies *Candida albicans* IP 48.72, *Candida albicans* IP 1180.79, *Zygosaccharomyces rouxii* IP 2021.92, *Aspergillus niger* IP 1431.83, *Penicillium verrucosum* IP 1232.80 are used. *Salmonella typhimurium* strains TA 98, TA 100, TA 102, TA 1535, and TA 1538, are used in genotoxic studies. All fungal strains were purchased from Institute Pasteur of Paris, France and bacterial strains were purchased from the American Type Culture Collection, USA.

Antimicrobial activities:

The antibacterial activity of the selected compounds was determined using the agar plate dilution method in accordance with National Committee for Clinical Laboratory Standards document M7-A5 for aerobic bacteria (NCCLS, 2001). Chemical compounds were dissolved in dimethyl sulphoxide (DMSO), and serial 2-fold dilutions were added to molten Mueller-Hinton Agar (Oxoid) to produce the concentration range of 4-512 µg/mL. After slight cooling and drying of the plates, a steer replicator was used to place aliquots containing approximately $5x10^4$ colony forming units per drop per plate for strains (*Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853). Plates were incubated at 35°C and the number of colonies recorded after 18 hour. The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which there was no growth, a faint haze, or < 3 discrete colonies. Plates were read in duplicate, and the higher MIC value was recorded.

The MIC concentrations of the fungal isolates were studied based on the same agar dilution method with the following modification: Sabouraud Dextrose Agar (Oxoid) was used instead of Mueller-Hinton Agar (MHA). The plates were spot inoculated with 5 µL (10^6spore/mL). The inoculated plates were incubated at 35°C for 48 hours, others were incubated at 30°C for 7 days (Gulluce *et al*, 2003).

Cytotoxic activity:

The tested compounds are prepared in three concentrations, 1, 10, and 100µg/mL. The Brine shrimp larvae (*Artemia Salina Leach*) were hatched in seawater for 24-48 hours before use in the test. One drop DMSO, 5 ml of seawater, and 10 brine shrimp were added to each vial. The dead ones were counted after 24 hours. The LD_{50} was calculated (Meyer *et al*, 1982).

Mutagenicity assay:

The basic procedure was the general assay (plate incorporation assay) described by Maron and Ames (Maron and Ames, 1983). The experiments were performed with *Salmonella typhimurium* strains TA 98, TA 102, TA 1535, and TA 1538. 100 ml for an overnight culture of bacteria and 500 µL of sodium phosphate (0.2 M, pH 7.4) was added to 2 mL aliquots of top agar (supplemented with 0.5 mM L-histidine and 0.5 mM D-biotin) containing different concentrations of the three compounds (2mg/mL-0.01 mg/mL). Positive and negative controls were used in each assay. The resulting complete mixture was poured over the minimal agar plate prepared as described by Maron and Ames (Maron and Ames, 1983). The plates were incubated at 37°C for 48 hours and the revertant bacterial colonies of each plate were counted.

Results and Discussion

Synthesis of the compounds:

The reactions of acid hydrazides (1a-d) with 2-acetylcyclohexanone gave 1-substituted-3-methyl-4,5,6,7-tetrahydrocyclohexanpyrazoles (2a-d). The reactions were carried out in a slightly acidic medium in ethanol under reflux. The reactions proceeded smoothly to give good yields of the corresponding fused ring pyrazoles. In similar fashion, hydrazides 1(a-d) readily reacted with 2-acetylcyclopentanone to give 1-substituted-3-methyl-4,5,6-trihydrocyclopentanepyrazoles (3a-d). Names, yields, melting points and IR spectroscopic data of these compounds are shown in Table 1.

The structures of the synthesized compounds (2a-d) and (3a-d) were confirmed based on their ^{1}H-NMR, ^{13}C-NMR and mass spectrometry (MS) as shown in Table 2.

Antimicrobial activity:

The antimicrobial activities of the selected compounds are shown in Table 3. Compound 3c was active against gram-positive microorganisms *Staphylococcus aureus*, and *Enterococcus faecalis*, as well as against gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* at concentration of 512μg/mL. The compound 3d was active only against gram-positive microorganisms at the same concentration, whereas the compound 1c was inactive against both types of microorganisms.

The results shown in Table 4 indicate that compound 3c was active against the two strains of *Candida albicans* and *Aspergillus niger* (128 μg/mL), as well as against *Zygosaccharomyces rouxii* (64 μg/mL), but was inactive against *Penicillium verrucosum*.

Compound 3d was active against the two strains of *Candida albicans* (256 μg/mL), and against *Zygosaccharomyces rouxii* (128 μg/mL), but was inactive against *Aspergillus niger* and *Penicillium verrucosum*. Despite the fact that the difference in the structure between compounds 3 and 2 is only minor and lies in the ring size of the fused ring system, it has been found that compound 2c was inactive against the fungal strains tested. Though it is difficult to rationalize this difference in activity it is probably due to the difference in structure. The results of the antimicrobial activity studies indicate that the incorporation of the furoyl group to 1-substituted-3-methyl-4, 5, 6, 7-tetrahydrocyclopentapyrazoles might improve their antimicrobial activities more than thienyl group.

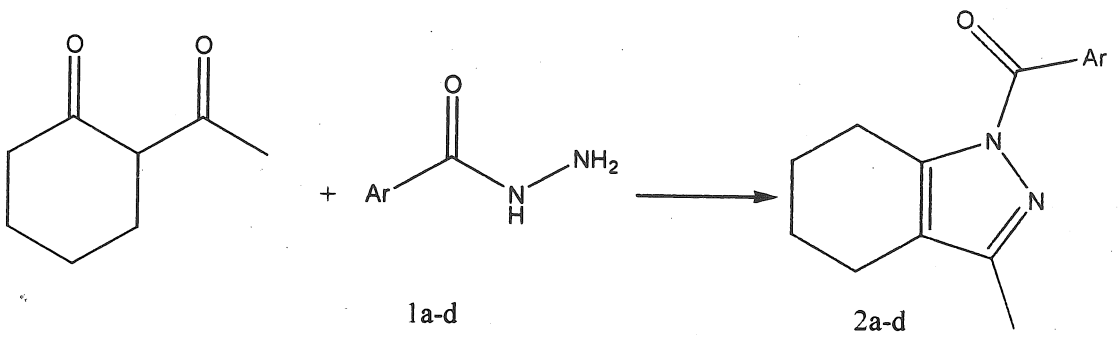
Cytotoxic activity:

The brine shrimp bioassay was used as screening test to determine the cytotoxicity of the three chemical compounds. The results are listed in Table 5. The two compounds 3c and 3d were found to be inactive with a median lethal concentration (LC_{50}) of more than 1000 μg/mL, the compound 2c was considered as a low active (LC_{50} < 1000 μg/mL) at high concentration (Gulluce *et al*, 2003). The brine shrimp bioassay indicates that all the compounds tested were found to be inactive as cytotoxic agents.

Mutagenic activity:

The results of the mutagenic activities were determined using *Salmonella typhimurium* strains TA 1535 and TA 100 to detect a base- pair substitution, and the strains TA 1537 and TA 98 to detect frameshift mutation. However the strain TA 102 was used as nonsense mutation detector. The results showed that none of these compounds have genotoxic properties in any of the *Salmonella typhimurium* strains tested (Kristien and Errol, 2000).

Scheme 1:



1a-d

2a-d

Compound	Ar
1, 2 a	p-$CH_3OC_6H_4$-
b	o-$CH_3OC_6H_4$-
c	2-furyl
d	2-thienyl

Table 1. Names*, yields, melting points and IR data of compounds 2(a-d) and compounds 3(a-d).

Comp. No.	Compound Name	Yield %	M.P. (°C)	IR (cm^{-1}) KBr (disc)
2a	1-(4-methoxybenzoyl)-3-methyl-4,5,6,7-tetrahydrocyclohexapyrazole	74	68-70	1671 (C=O) 1599 (C=N)
b	1-(2-methoxybenzoyl)-3-methyl-4,5,6,7-tetrahydrocyclohexapyrazole	63	84-86	1700 (C=O) 1603 (C=N)
c	1-(2-furoyl)-methyl-4,5,6,7-tetrahydrocyclohexapyrazole	67	132-133	1676 (C=O) 1559 (C=N)
d	1-(2-thienoyl)-3-methyl-4,5,6,7-tetrahydrocyclohexapyrazole	76	104-106	1666 (C=O) 1602 (C=N)
3a	1-(4-methoxybenzoyl)-3-methyl-4,5,6-trihydrocyclohpentapyrazole	59	87-89	-
b	1-(2-methoxybenzoyl)-3-4,5,6-trihydrocyclohpentapyrazole	55	102-103	-
c	1-(2-furoyl)-3-methyl-4,5,6-trihydrocyclohpentapyrazole	56	148-150	1671 (C=O) 1560 (C=N)
d	1-(2-thienoyl)-3-methyl-4,5,6-trihydrocyclohpentapyrazole	65	124-125	1661 (C=O) 1594 (C=N)

* All new compounds gave satisfactory elemental analysis of C, H and N.

Table 2. ^{1}H-NMR, ^{13}C-NMR and mass spectral data for compounds 2(a-d) and compounds 3(a-d)

Comp. No.	^{1}H-NMR δ(ppm) $CDCl_3$-TMS	^{13}C-NMR ($CDCL_3$/TMS) δ(ppm)	(m/z, rel.int. %)
2a	8.02 (d, 2H, J = 9Hz, Ar); 6.95 (d, 2H, J = 9Hz, Ar); 3.88 (s, 3H, O-CH_3); 2.70-2.50 (m, 7H, $2CH_2$, CH_3); 1.80-1.76 (m,4H, $2CH_2$)	---	270 (M^+, 69); 135 ($CH_3OC_6H_4CO$+, 100); 107 ($CH_3OC_6H_4^+$, 47)
b	7.46-6.85 (m,4H, Ar); 3.80 (s, 3H, O-CH_3); 2.65-2.43 (m, 7H,$2CH_2$, CH_3); 1.76-1.72 (m, 4H, $2CH_2$)	---	239 (M^+-OCH_3, 95); 135 ($CH_3OC_6H_4^+$, 100)
c	7.78 (d, 1H, Ar); 7.61 (d, 1H, Ar); 6.48 (t, 1H, Ar); 2.60 (t, 2H, J=6Hz, CH_2); 2.42 (s, 3H, CH_3); 2.37 (6, 2H, J=6Hz, CH_2); 1.73-1.62 (m, 4H, $2CH_2$)	157.0, 154.2, 147.2, 146.0, 139.6, 123.4, 118.9, 112.2, 23.8, 23.0, 22.9, 20.0, 12.5	230 (M^+, 57); 202 (M^+-N_2, 44); 135 (M^+-$C_4H_3OCO^+$, 85) 95 ($C_4H_3OCO^+$, 100)
d	8.19 (d, 1H, Ar); 7.59 (d, 1H, Ar); 7.02 (t, 1H, Ar); 2.61 (t, 2H, J=6Hz, CH_2); 2.42 (s, 3H, CH_3); 2.35 (t, 2H, J=6Hz, CH_2); 1.72-1.64 (m, 4H, $2CH_2$)	160.7, 153.7, 139.3, 137.4, 136.4, 134.3, 126.8, 119.3, 23.7, 23.0, 22.9, 20.1, 12.7	246 (M^+, 43); 135 (M^+-C_4H_3SCO, 81); 111 (C_4H_3SCO+, 100)
3a	8.02 (d, 2H, J=9, Ar); 6.95 (d, 2H, J=9, Ar); 3.86 (s, 3H, O-CH_3); 3.10-3.05 (m, 2H, CH_2); 2.60-2.54 (m, 4H, $2CH_2$); 2.23 (s, 3H, CH_3)	165.4, 163.1, 153.8, 148.1, 133.9, 130.4, 124.3, 113.3, 55.4, 30.2, 27.4, 22.1, 13.1	256 (M^+, 75); 135 ($CH_3OC_6H_4CO^+$, 100); 107 ($CH_3OC_6H_4^+$, 55)
b	7.47.6.91 (m, 4H, Ar); 3.82 (s, 3H, O-CH_3); 2.90-2.83 (m, 2H, CH_2); 2.57-2.48 (m, 4H, $2CH_2$); 2.19 (s, 3H, CH_3)	---	225 (M^+ -OCH_3, 97); 135 ($CH_3OC_6H_4^+$, 100)
c	7.93 (d, 1H, Ar); 7.63 (d, 1H, Ar); 6.50 (t, 1H, Ar); 3.00-2.97 (m, 2H, CH_2); 2.46-2.16 (m, 4H, $2CH_2$); 2.16 (s, 3H, CH_3)	156.0, 153.7, 148.8, 147.5, 145.2, 130.7, 123.6, 112.3, 130.7, 123.6, 112.3, 30.2, 27.2, 22.0, 13.1	216 (M^+, 35); 121 (M^+ - C_4H_3OCO, 25); 95 ($C_4H_3OCO^+$, 100)
d	8.28 (d, 1H, Ar); 7.64 (d, 1H, Ar); 7.07 (t, 1H, Ar); 3.02-2.97 (m, 2H, CH_2); 2.50-2.46 (m, 4H, $2CH_2$); 2.20 (s, 3H, CH_3)	158.8, 153.4, 148.4, 137.6, 136.4, 133.1, 131.1, 127.1, 30.3, 27.2, 22.1, 13.1	232 (M^+, 41); 121 (M^+ - C_4H_3SCO, 29); 111 ($C_4H_3SCO^+$, 100)

Table 3. Antibacterial activity test results

Microorganism	MIC 512 µg/mL		
	3c	2c	3d
Staphylococcus aureus ATCC 29213	+	-	+
Enterococcus faecalis ATCC 29212	+	-	+
Escherichia coli ATCC 25922	+	-	-
Pseudomonas aeruginosa ATCC 27853	+	-	-

Table 4. Antifungal activity test result

Microorganism	MIC (µg/mL)		
	3c	2c	3d
Candida albicans IP 48.72	128	–	256
Candida albicans IP 1180.79	128	–	256
Zygosaccharomyces rouxii IP 2021.92	64	–	128
Aspergillus niger IP 1431.83	128	–	-
Penicillium verrucosum IP 1232.80	–	–	–

Table 5. Brine shrimp bioassay and cytotoxicity results.

Number of shrimps killed at (μg/mL)				
Chemical	100	10	1	LD_{50}*
3c	12	6	4	>100
2c	16	10	7	754.7
3d	14	6	3	>1000

* LD50 is the dose that kills half (50%) of the shrimps tested (LD50 = "lethal dose")

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