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Synthesis and wound healing activity of substituted phenylhydrazono-*N*-methylacetoacetamides

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phenylhydrazono-*N*-methylacetamides / wound healing activity

Introduction

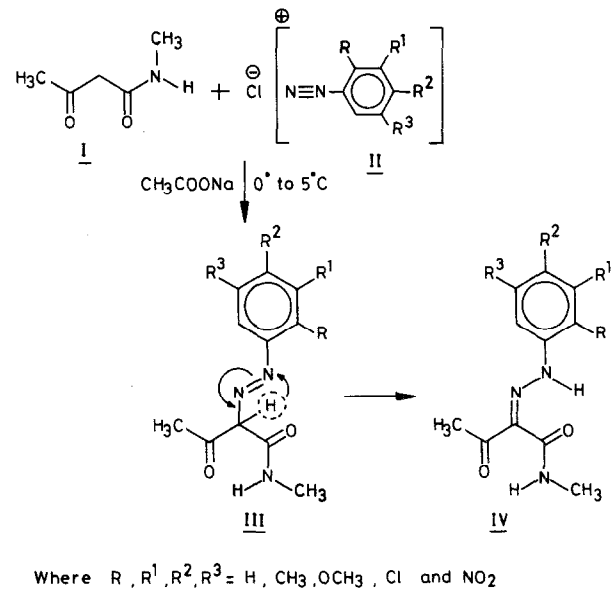
As part of our research work on the design of biologically active molecules [1], we have now synthesized some new substituted phenylhydrazono-*N*-methylacetoacetamides **IV**. In the previous communication [2], we reported the synthesis and X-ray investigation of 2(4-chlorophenylhydrazono)*N*-methylacetoacetamide **7**. The promising wound healing activity shown by the compound **7** in table I, prompted us to synthesize a number of compounds **IV** for an expanded study of wound healing activity. There is no report on this class of compounds possessing wound healing activity in normal and diabetic rats.

Chemistry

In the present investigation, a variety of substituted phenylhydrazono-*N*-methylacetoacetamides **IV** have been synthesized by general procedure [2] by coupling substituted benzenediazonium chloride **II** with *N*-methylacetoacetamide **I** in presence of sodium acetate and ethanol. The rearrangement of phenylazo-*N*-methylacetoacetamide **III** to the more stable phenylhydrazono-*N*-methylacetoacetamide **IV** is given in scheme 1. A common feature to all these derivatives is that during the synthesis, a diazonium rearrangement occurs. The products obtained were invariably bright coloured solids. All the compounds in table I were characterized by spectroscopic techniques and elemental analysis.

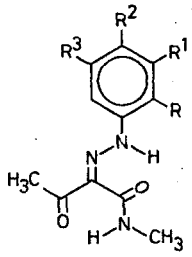
Results and discussion

All compounds (**1–15** in table I) were tested for excision, incision and dead space wounds. The study revealed that in normal healthy rats, wound healing was significantly enhanced by the compounds **9**, **10** and **12** having 3,4-dichloro, 2-nitro-4-chloro and 4-nitro substitution on the phenyl ring. Table II shows that the rate of wound contraction was total on the 16th day for the compounds **5**, **6**, **7**, **11** and **13**, whereas for the compounds **9**, **10** and **12**, the total wound contraction was on the 10th day. The compounds **10**, **11** and **12** having nitro substitution at 2- and 4-position on phenyl ring are found to be effective in the wound healing. The compounds **7**, **9**, **10** and **12** having 4-chloro, 3,4-dichloro, 2-nitro-4-chloro and



Scheme 1.

Table I. Characterization of substituted phenylhydrazono-*N*-methylacetoacetamides **1–15**.



S.No.	R	R^1	R^2	R^3	Mol. formula* (Mol. wt.)	m.p.(°C)	Recryst.[a] solvent	Yield (%)
1	H	H	H	H	$C_{11}H_{13}N_3O_2$ (219.35)	139-140	A	77.6
2	H	CH_3	H	H	$C_{12}H_{15}N_3O_2$ (233.26)	109-110	A	85.7
3	H	H	CH_3	H	$C_{12}H_{15}N_3O_2$ (233.26)	119-120	A	83.6
4	H	H	OCH_3	H	$C_{12}H_{15}N_3O_3$ (249.26)	115-116	B	80.2
5	Cl	H	H	H	$C_{11}H_{12}ClN_3O_2$ (253.68)	129-130	C	80.8
6	H	Cl	H	H	$C_{11}H_{12}ClN_3O_2$ (253.68)	146-147	C	94.6
7	H	H	Cl	H	$C_{11}H_{12}ClN_3O_2$ (253.68)	140-141	A	90.0
8	Cl	Cl	H	H	$C_{11}H_{11}Cl_2N_3O_2$ (288.11)	165-166	A	83.3
9	H	Cl	Cl	H	$C_{11}H_{11}Cl_2N_3O_2$ (288.11)	194-195	D	85.0
10	NO_2	H	Cl	H	$C_{11}H_{11}ClN_4O_4$ (298.68)	229-230	A	93.9
11	NO_2	H	H	H	$C_{11}H_{12}N_2O_4$ (264.24)	202-203	B	94.6
12	H	H	NO_2	H	$C_{11}H_{12}N_4O_4$ (264.24)	180-182	B	87.0
13	NO_2	H	OCH_3	H	$C_{12}H_{14}N_4O_5$ (294.26)	220-221	A	87.0
14	OCH_3	H	NO_2	H	$C_{12}H_{14}N_4O_5$ (294.26)	228-229	D	88.3
15	OCH_3	H	H	NO_2	$C_{12}H_{14}N_4O_5$ (294.26)	278 (decomp.)	C	95.0

*All compounds were analyzed for C, H and N and the results obtained were within ± 0.4 of the theoretical values.

[a]A = ethanol, B = ethanol water, C = methanol, D = acetone.

Table II. Effect of substituted phenylhydrazone-*N*-methylacetoacetamides on various parameters in wound healing. Data are mean ± SE, NT, not tested.

Compounds	Excision wound healing in normal rats wound contraction in (%)[a]						Excision wound healing in diabetic rats wound contraction in (%)[a]					
	4th day	8th day	12th day	16th day	20th day	Period of epithelization (days)	4th day	8th day	12th day	16th day	20th day	Period of epithelization (days)
Control	27 ± 3.0	62 ± 2.9	83 ± 1.1	92 ± 1.2	healed	20 ± 2.0	22 ± 9.8	64 ± 0.8	88 ± 0.7	97 ± 2.4	-	30 ± 2.0
5	44 ± 3.6	81 ± 1.8	91 ± 0.9	healed	-	16 ± 2.0	NT	NT	NT	NT	NT	NT
6	58 ± 4.1	85 ± 0.4	92 ± 1.0	healed	-	16 ± 1.6	7 ± 2.0	60 ± 1.0	87 ± 2.7	-	-	25 ± 3.0
7	62 ± 1.8	84 ± 1.4	94 ± 2.4	healed	-	16 ± 2.0	17 ± 6.0	63 ± 3.0	92 ± 3.6	96 ± 3.6	-	27 ± 2.0
9	50 ± 1.1	88 ± 2.6	healed	-	-	10 ± 3.0*	14 ± 3.1	55 ±10.0	88 ± 3.0	-	-	21 ±3.0**
10	39 ± 4.7	89 ± 0.2	healed	-	-	10 ± 3.0*	4 ± 1.5	76 ± 0.8	88 ± 0.6	91 ± 2.0	-	21 ± 3.0**
11	11 ± 2.7	52 ± 2.4	81 ± 1.4	healed	-	16 ± 2.0	NT	NT	NT	NT	NT	NT
12	41 ± 6.0	87 ± 2.3	healed	-	-	10 ± 2.0*	14 ± 7.4	82 ± 3.6	93 ± 0.7	96 ± 0.5	-	21 ± 3.0**
13	15 ± 2.6	50 ± 4.7	86 ± 0.7	healed	-	16 ± 3.0	NT	NT	NT	NT	NT	NT
14	13 ± 2.8	60 ± 2.3	78 ± 2.4	81 ± 0.6	healed	21 ± 4.0	NT	NT	NT	NT	NT	NT
15	12 ± 3.0	66 ± 1.5	84 ± 0.7	89 ± 0.8	healed	21 ± 1.4	NT	NT	NT	NT	NT	NT

*$P < 0.001$ compared to control; ** $P < 0.01$.

4-nitro groups on phenyl ring showed a significant decrease in wound contraction in diabetic rats on 21st day.

The compounds **13**, **14** and **15** which were prepared to examine the effects of methoxy substitution on 2- and 4-position of phenyl ring were found to be less potent in wound healing.

In the incision wound, the tensile strength of 8th day old wound was found to be 542 ± 13.6 g in rats treated with compound **6**, while in the control it was found to be 383 ± 10 g. It is observed from these results that the compounds **6**, **7** and **12** significantly promoted gain in tensile strength compared to the control (table III).

In dead space wounds, the mean granuloma weight was 63.8 ± 7.3 mg/100 g body weight in control animals (table III). The corresponding values in animals treated with compounds **10** and **12** were significantly lower 34.9 ± 4.1 and 35.7 ± 7.3 mg/100 g body weight respectively indicating that the compounds **10** and **12** inhibited granulation on the implanted cotton pellet.

The present study suggests, therefore, that substituted phenylhydrazono-*N*-methylacetoacetamides **IV** have differential action on different phases of wound healing. These compounds suppress granulation, promote wound contraction and gain in tensile strength.

Table III. Incision and dead space wound studies. Results compared with control. NS = not significant.

Compound No.	Incision wound Tensile strength (g) Mean ± SE	Significance P^a value	Dead space wound Granuloma weight mg/100 g of body weight Mean ± SE	Significance P^a value
6	542 ± 13.6	< 0.001	64.4 ± 5.1	NS
7	516 ± 11.0	< 0.001	58.6 ± 3.0	NS
9	508 ± 11.5	< 0.001	62.0 ± 2.7	NS
10	510 ± 12.4	< 0.001	34.9 ± 4.1	< 0.01
12	539 ± 24.0	< 0.001	35.7 ± 7.3	< 0.01
Control	383 ± 9.5		63.8 ± 7.3	

Experimental protocols

Chemistry

Melting points were taken using an Edmund Bühler melting point apparatus and are uncorrected. Melting points and recrystallization solvents are shown in table I. The IR spectra

were recorded on Perkin–Elmer model 283 B spectrophotometer in potassium bromide pellet. Mass spectra were recorded on VG 7070 H mass spectrometer at 70 eV. The NMR spectra were determined in a Jeol FX 90 Q FT NMR spectrometer. Chemical shifts are reported in parts per million δ relative to Me_4Si as an internal standard.

*General procedure for the synthesis of substituted phenylhydrazono-N-methylacetoacetamide [**1–15**, table I]*

Substituted aniline (0.1 mol) was dissolved in diluted hydrochloric acid (80 ml in 400 ml of water) and the solution was cooled to 0°C in an ice-salt bath with vigorous stirring. A cold solution of sodium nitrite (0.1 mol) in 100 ml of water was added portionwise over a period of 15 min. The solution was well stirred during the diazotisation and the mixture was kept at a temperature of 0 to 5°C by addition of a little crushed ice from time to time. The liberated nitrous acid was tested with potassium iodide-starch paper. *N*-Methyl acetoacetamide (0.1 mol) was dissolved in 15 ml of ethanol and sodium acetate (0.1 mol) in 40 ml of water was added. The mixture was cooled to 0°C with vigorous stirring. To this cold diazonium chloride solution was added portionwise with stirring. The reaction mixture was kept at room temperature for 2–3 h. A bright coloured product was separated, filtered, washed with water, dried and recrystallized from suitable solvent, as shown in table I. Compound **12** in table I showed characteristic IR bands at 3300 (NH), 1665 (C=O), 1610 (C=C), 1595 (C=N), 1515 (CONH), 1340 (NO_2) cm^{-1}. ^1H NMR ($CDCl_3$) δ: 2.5 (s, 3H, CH_3); 2.90–2.95 (d, 3H, N-CH_3); 7.30–7.40 (m, 4H, ArH); 8.32 (s, 1H, NH). MS: m/z 264 (M^+, 80), 43 (100).

Biological activity

The compounds given in table I were screened for wound healing activity. Three models of wounds have been employed *viz.* (i) excision, (ii) incision and (iii) dead space wounds. All test compounds were administered orally in 2% gum acacia suspension at a dose of 100 mg/kg. For excision wound model, test compounds were applied locally as 2% ointment (petroleum jelly *ie* soft paraffin or paraffinum molle) in different groups of animals. Gum acacia and petroleum jelly were used as the standard for oral route and local application.

Wistar rats of either sex weighing about 150–200 g were used. The animals were divided into different groups, each group contained 6 animals of either sex. Animals were starved overnight and the wound was inflicted under light ether anaesthesia. The wounds were mopped and left undressed by the method of Lee [3].

Excision wounds

On the depilated back of each rat, excision wound was inflicted by cutting away a circular piece (3.80 x 1 cm^2) full thickness skin of a pre-determined area according to the method of Morton and Melon [4]. These wounds were employed to monitor the rate of wound contraction and epithelization time. The test compound 2% ointment (preparation with petroleum jelly) was applied locally to the wound once daily. Measurements of wound area in terms of shape, size and scar were recorded on every 4th day. Reduction in wound area was expressed as percent of original wound size (3.80 x 1 cm^2). Epithelization time was measured in days, from day 0 (wounding day) till the day escher fell off with no raw wound left behind.

Incision wounds

On the depilated back of each rat two 6 cm long paravertebral incisions were made, cutting through the full thickness of the skin. After moping the wounds and ensuring haemostesis, the wounds were closed by interrupted sutures 1 cm apart. The test compounds were administered orally per day to rats at 100 mg/kg body weight in 0.5% gum acacia suspension from the date of wounding. The sutures were removed on the 7th post-wounding day and tensile strength measured on 8th day by the method of Lee [3].

Dead space wounds

Sterilised cotton pellets (10 mg each) were implanted subcutaneously one in each axilla and groin of male albino rats according to method of Turner [5]. On the 8th day, the granuloma grown on the cotton pellets were carefully dissected out, dried at 60°C overnight and weighed. The dry weights so obtained were expressed in mg/100 g body weight as suggested by Dipasquale and Meli [6]. The test compounds were administered orally per day (100 mg/kg) to each of the treated animals for 7 days from the day of the implantation.

Wound healing in diabetic rats

Wistar rats of either sex weighing 200 to 250 g were used to induce diabetes by administering streptozotocin at a dose of 70 mg/kg by the procedure described by Srivastava [7]. The animals were starved overnight. The rats were given pellets and water *ad libitum* and blood sugar levels were checked after 4 days. Diabetic rats with blood sugar levels above 300 mg/dl were used in this experiments. Excision wound procedure and treatment are the same as described earlier.

None of the rats had any significant change in their body weight during the period of study.

Acute toxicity

Acute toxicity was studied in rats and mice. The test compounds were administered orally and LD_{50} in terms of mg/kg was calculated by the method described by Ghosh [8]. In all the compounds, the LD_{50} was found to be greater than 2000 mg/kg, *po*.

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