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Synthesis of 4-methyl (6,7-*b*-tetrahydrobenzofurano) coumarin and its contraception like properties in male rabbits (*Oryctolagus cuniculus*)

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Abstract. Administration of 4-methyl (6,7-*b*-tetrahydrobenzofurano) coumarin, 20 mg/kg/alternate day, for a period of 40 days caused degenerative changes in the testes of male rabbits. Inhibition of spermatogenesis was achieved at primary spermatocyte stage level. Total protein, sialic acid and glycogen contents of the testes, epididymis and seminal vesicle were significantly reduced while the testicular cholesterol was elevated in the 4-methyl coumarin treated animals. Serum cholesterol, phospholipid, triglyceride, NEFA, were elevated. Antispermatogenic activity of 4-methyl coumarin is discussed.

Keywords. 4-methyl coumarin ; inhibition of spermatogenesis ; sialic acid ; antiandrogenicity.

1. Introduction

Simple aliphatic compounds like triethylene melamine exhibit antifertility properties (Jackson 1964), characterized by damage of spermatogonia and germinal epithelium (Steinberger 1962). Lednicer *et al* (1965) prepared a number of 3,4-diaryl coumarins sterically related to 1,2-diaryl indene and showed that some of these possess antifertility activity.

Marked antifertility activity was also observed in the compounds incorporating triarylethylene and also in 3,4-diphenyl, 1,2,3,4-tetranaphthalene when the hydroxy or alkoxy group was introduced. Mishra and Agrawal (1977) synthesized several new *bis* and *di* (or 4'-coumarynil-oxyalkanes) coumarins and later tested them for possible antifertility activity.

Realising the importance of benzofuran, coumarin and cyclohexanol derivatives (Tyagi *et al* 1979) as antifertility agents, it was considered worthwhile to design molecule incorporating benzofuran, coumarin and cyclohexanol moieties.

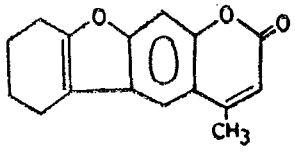
2. Experimental

In this direction 2-halo-cyclohexane was condensed with 7-hydroxy, 4-methyl coumarin. 4-methyl-7-hydroxy coumarin (4·4 g, 0·025 M), 2-bromocyclohexanone (5·31 g ; 0·03 M), anhydrous K_2CO_3 (8·0 g) and dry acetone (80·0 ml) were taken in a round bottom flask, fitted with a refluxed condensor. The reaction mixture, after refluxing for 60 hr, was cooled and filtered.

The solvent was distilled off under vacuum. The crude product was crystallized with 95% ethanol. A white crystalline solid compound was obtained. The purity was ascertained by TLC (m.p. 160° C ; yield 3·50 g, 55%, Rf 0·89). Its derivative with 2,4-dinitrophenyl hydrazine was prepared (m.p. 169–170° C).

NMR spectrum was obtained in TFA using TMS as internal standard. NMR spectrum indicated the presence of singlet for 3 protons at δ 2·4. In the spectrum complicated pattern for 8 proton in the range of δ 1·1 to 2·1, a broad singlet for a proton at δ 6·2 and 2–protons in the aromatic region (δ 6·75 to 7·45) were observed. The NMR spectrum accounted well for the presence of 14 protons. The presence of a singlet at δ 2·4 was due to the C–methyl (C-CH_3) groups attached to position 4 in coumarin system. Methyl group being attached to an aromatic ring and the olifinic bond, conjugated to a carbonyl group alongwith its presence in a pyron ring gave a broad singlet to the down field at δ 2·4. The presence of a broad singlet at δ 6·2 for a proton accounted for the presence of olifinic proton. The presence of only two aromatic protons indicated fusion of cyclohexane ring to the ortho position of –OH group. The presence of 8-protons at much higher field (δ 1·1 to 2·1) indicated the presence of 4-methylene groups and the absence of methane protons at the same time. This clearly indicated the presence of cyclohexane system.

Based on the observations IR/NMR the structure of the compound I was assigned as 4-methyl (6,7-*b*-tetrahydrobenzofurano) coumarin (Tyagi *et al* 1980).



4–methyl (6,7–*b*–tetrahydrobenzofurano) coumarin

3. Material and methods

Twenty healthy adult male rabbits were used in the experiment and were divided into groups as outlined in table 1. Ten rabbits comprising group 2 were treated with 4-methyl (6,7-*b*-tetrahydrobenzofurano) coumarin 20 mg/kg/each alternate day s.c. for 40 days. An equal number of rabbits received the vehicle alone and served as control. After the completion of the final dose of 4-methyl coumarin, rabbits were sacrificed with nembutal anaesthesia. Blood was withdrawn through cardiac puncture and serum analysed.

Table 1. Changes in the weight of testis, epididymis and adrenal glands together with seminiferous tubule and Leydig cell nuclear diameter of rabbit after 4-methyl (6,7-*b*-tetrahydrobenzofurano) coumarin treatment.

	Body weight (kg)	Testes	Epididymis	Adrenal	Seminiferous tubule diameter (μm)	Leydig cell nuclear diameter (μm)
			mg/kg			
Control (10)	$1{\cdot}4 \pm 0{\cdot}3$	1931 ± 108	780 ± 64	210 ± 35	$175 \pm 2{\cdot}4$	$6{\cdot}00 \pm 0{\cdot}17$
4-methyl coumarin (10)	$1{\cdot}3 \pm 0{\cdot}2$*	1336 ± 146*	470 ± 43*	278 ± 26†	$114 \pm 1{\cdot}0$**	$5{\cdot}16 \pm 0{\cdot}148$*

4-methyl coumarin *versus* control : ** $P < 0{\cdot}001$ *$P < 0{\cdot}02$ †NS (Not significant) ; All figures $\pm$ S.E.M. Figures in parenthesis represent the number of animals examined.

Final body weight of each animal from both groups were recorded. Testoes, epididymis, seminal vesicle and adrenal glands were dissected free of fat. Right testis and epididymis were fixed in Bouin's fluid. 6 μm sections were prepared and stained with haematoxylin and eosin. Left testis, epididymis, seminal vesicle and adrenal glands were frozen and the total protein, sialic acid, testicular cholesterol, glycogen, acid phosphatase and adrenal ascorbic acid were later determined (Lowry *et al* 1951 ; Warren 1959 ; Montgommery 1957 ; Fiske and Subbarow 1925 ; Roe and Kuether 1943). Quantitative estimation of cholesterol was made according to the Libermann–Burchard method (Oser 1965). Serum was analysed for cholesterol, phospholipids, triglyceride, non-esterified free fatty acid and serum proteins (Varley 1969). The transaminase enzyme activity (SGPT) was determined according to Mohun and Cook (1957).

One hundred seminiferous tubules appearing circular in sections were traced with camera lucida at $\times 80$. Two perpendicular diameters of each group tracing were measured and expressed in terms of mean tubular diameters. Student's 't' test was applied for comparing means. The measurements of the diameters of 100 Leydig cell nuclei were carried out from the sections of testes with camera lucida drawings at $\times 800$.

The LD_{50} of 4-methyl (6,7-*b*-tetrahydrobenzofurano) coumarin worked out in white albino rat comes out to be 200 mg/kg.

Dizziness and paralytic conditions were the main symptoms observed.

4. Results

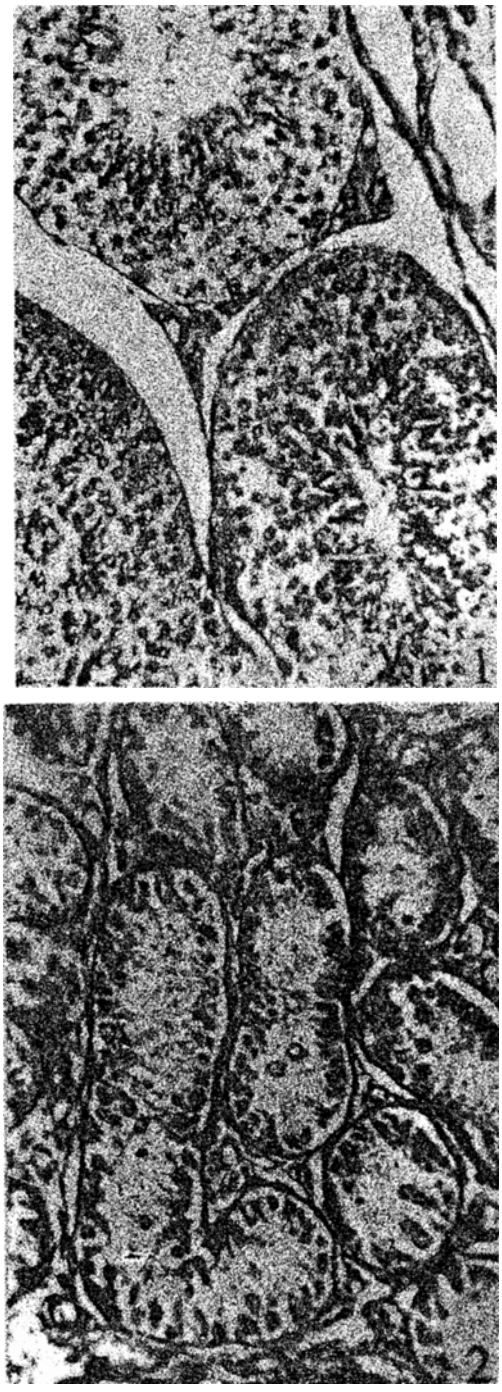
4.1. *Body weight and organ weight* (*table* 1)

The body weight of the rabbits treated with 4-methyl coumarin was insignificantly reduced. The testicular weight and epididymal weight exhibit significant reduction in the 4-methyl coumarin-treated animals when compared with controls.

Table 2. Changes in protein, sialic acid, glycogen, cholesterol and adrenal ascorbic acid content of testis, epididymis and seminal vesicle of rabbits after 4-methyl (6,7-*b*-tetrahydrobenzofurano) coumarin treatment.

	Protein			Sialic Acid			Acid Phosphatase			Glycogen	Cholesterol	Ascorbic acid adrenal
	T	E	SV	T	E	SV	T	E	SV	T	T	
	μg/mg						μg Pi/mg Tissue/hr			mg/g		mg/μg
Control	230 ± 18	179 ± 21	177 ± 6	5·6 ± 0·3	4·8 ± 0·2	6·7 ± 0·3	2·68 ± 0·3	2·24 ± 0·3	2·6 ± 0·3	2·1 ± 0·3	3·6 ± 0·6	5·04 ± 0·51
4-methylcoumarin	110 ± 4*	110 ± 7†	119 ± 5*	3·8 ± 0·1*	3·4 ± 0·1*	3·9 ± 0·2*	1·63 ± 0·2*	1·33 ± 0·2*	1·5 ± 0·1*	0·9 ± 0·1†	7·2 ± 0·5*	3·5 ± 0·1†

T = Testis ; E = Epididymis ; SV = Seminal vesicle ; 4-Methyl coumarin *versus* control : * $P < 0·01$ † $P < 0·05$ All figures ± SEM
Biochemical estimations : Means of six determinations.



Figures 1–2. 1. Testis of a control rabbit showing various stages of spermato genesis × 160 HE. **2.** After 4-methyl coumarin treatment. Note the loss of various cell stages × 160 HE.

4.2. *Histological changes*

4.2a. *Testes* : In the rabbits treated with 4-methyl coumarin, the seminiferous tubule diameter and Leydig cell nuclear diameter decreased significantly (table 1). Spermatogenesis was arrested at primary spermatocyte stage. The changes consisted of loss of spermatids and spermatozoa (figures 1, 2). Sertoli cells were normal.

4.2b. *Epididymis* : Histological examination of the epididymis of 4-methyl coumarin-treated rabbits showed that the epithelium was normal and the lumen of caput epididymis was filled with debris. Cauda epididymis and ductus deferens were devoid of spermatozoa.

4.3. *Biochemical changes*

4.3a. *Protein* : The total protein contents of testis, epididymis and seminal vesicle were significantly lower in the rabbits treated with 4-methyl coumarin in comparison with controls (table 2).

4.3b. *Sialic acid* : The level of sialic acid was significantly decreased in the testis, epididymis and seminal vesicle of 4-methyl coumarin-treated rabbits (table 2).

4.3c. *Acid phosphatase* : Acid phosphatase enzyme activity of the testis, epididymis and seminal vesicle was reduced significantly after 4-methyl coumarin treatment (table 2).

4.3d. *Glycogen* : The glycogen level of testes decreased significantly (table 2).

4.3e. *Cholesterol* : The total cholesterol of testis increased in treated animals (table 2).

4.3f. *Ascorbic acid* : The ascorbic acid contents of adrenal glands were low (table 2).

4.3g. *Serum analysis* : The decrease in serum protein of coumarin-treated animals was highly significant ($P < 0{\cdot}01$). No significant change was observed

Table 3. Serum analysis of rabbit after 4-methyl (6,7-*b*-tetrahydrobenzofurano) coumarin treatment.

	Protein	Cholesterol	Phospholipid	Triglyceride	NEFA	SGPT
			(mg/100 m)		mEq/L	Reitman Frankel units
Control	11420 ± 180	121·4 ± 10·2	130 ± 7·24	73 ± 4	0·244 ± 0·014	99 ± 10
4-methyl-coumarin	8030 ± 213*	152 ± 12†	176 ± 9**	116 ± 3*	0·370 ± 0·02*	109 ± 23†

4-methyl coumarin *versus* control : * $P < 0{\cdot}01$ ** $P < 0{\cdot}05$ † NS (Not significant). All figures ± SEM. Biochemical estimations : Means of six determinations,

in pyruvate transaminase activity, however an increase was recorded in the total cholesterol, phospholipid, triglyceride and non-esterified fatty acids (table 3).

5. Discussion

Compounds which suppress spermatogenesis include many chemical classes and modes of action (Jackson 1970). Little information is available concerning structure activity relationships and metabolism of these interesting compounds.

After 40 days of 4-methyl (6,7-*b*-tetrahydrobenzofurano) coumarin treatment resulted in disappearance of mature sperms and spermatids. These results are similar as observed after ethylene dimethane sulphonate (Jackson 1970), nitrofuran and α-chlorohydrin (Patanelli 1975). Decrease level of protein in the testes, epididymis and seminal vesicle of rabbits treated with 4-methyl (6,7-*b*-tetrahydrobenzofurano) coumarin suggest an inhibition of speramatogenesis and suppressed Leydig cell function. Podesta *et al* (1975) describe a relationship between the androgen sensitivity and protein synthesis, contents and concentration, in the epididymis. A decrease in the level of protein of epididymis reflects the antiandrogenic nature of the compound. Significant decrease in glycogen may affect protein synthesis and thus subsequently inhibit spermatogenesis.

Reduced acid phosphatase enzyme/sialic acid activity confirms the inhibitory role of 4-methyl coumarin on spermatogenesis in rabbit. Blackshow and Massey (1978) showed that the total and free biochemical acid phosphatase decreased during cryptorchidism. Peyre and Laporte (1966), Rajalakshmi and Prasad (1968) reported a fall in the sialic acid contents of cryptorchid testes/epididymis of castrated rats and intact langur monkeys (Braz *et al* 1979).

A significant increase in testicular/serum cholesterol after 4-methyl coumarin treatment have been considered physiologically important, since testicular cholesterol derived from blood cholesterol is used for testosterone production (Anderson and Dietschy 1977) and is the primary substrate for androgen biosynthesis (Dorfman *et al* 1963 ; Eik-Nes and Kekre 1963).

Serum protein was reduced while cholesterol was elevated. The phospholipids, triglycerides and non-esterified fatty acids were also increased in the rabbits following 4-methyl (6,7-*b*-tetrahydrobenzofurano) coumarin treatment, reflects an interference in the lipid metabolism. However, more work is in progress for the reversible action of the compound and shall be reported elsewhere.

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