Pergamon

Bioorganic & Medicinal Chemistry 6 (1998) 833–839

BIOORGANIC & MEDICINAL CHEMISTRY

Mechanism of Biochemical Action of Substituted 4-Methylbenzopyran-2-ones. Part I: Dioxygenated 4-Methyl Coumarins as Superb Antioxidant and Radical Scavenging Agents

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Received 25 April 1997; accepted 16 January 1998

Abstract—Twenty-three 4-methylcoumarins bearing different functionalities have been examined for the first time for their effect on NADPH-catalysed liver-microsomal lipid peroxidation with a view to establish structure–activity relationship. Dihydroxy- and diacetoxy-4-methylcoumarins produced dramatic inhibition of lipid peroxidation. 7,8-Diacetoxy-4-methylcoumarin and 7,8-dihydroxy-4-methylcoumarin were found to possess superb antioxidant and radical scavenging activities.

Introduction

Considerable progress has been made in recent years relating free radicals, especially reactive oxygen species in living cells to pathogenecity of various diseases[1,2] including hepatic and vascular diseases.[3,4] Efforts to discover antioxidants as useful drug candidates to combat these diseases are going on relentlessly. In this connection, minor dietary constituents, especially plant-based foods have come under serious scrutiny. Polyphenolic compounds have drawn greater attention compared to any other class of natural products to counter the ill effects of oxygen radicals. Among them the antioxidant and radical scavenging effects of flavones and their derivatives are highly cited.[5–7] Coumarins are quite similar to flavonoids in nature and are widely distributed in the plant kingdom, 4-methylcoumarins are implicated to have several beneficial pharmacological effects.[8–11] Our laboratory is engaged in studying the chemistry and biological effects of 4-methylcoumarin derivatives (Raj et al.).[12,13] In this report we have examined the effects of a large number of synthetic 4-methylcoumarin derivatives on liver microsomal lipid peroxidation and radical scavenging with a view to elucidate structure–activity relationship. 4-Methylcoumarins, having two hydroxy or two acetoxy groups in the benzonoid ring at positions *ortho* to each other, have shown very strong antioxidant and radical scavenging properties, better than those of α-tocopherol.

Materials and Methods

Chemicals

NADPH, ADP and trichloroacetic acid (TCA) were obtained from Sisco Research Laboratory (Mumbai, India). Tris, $FeCl_3$, thiobarbituric acid (TBA),

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0968-0896/98/$19.00
PII: S0968-0896(98)00043-1

dimethylsulphoxide (DMSO) and ascorbic acid of high purity were procured from local suppliers. Diphenyl picrylhydrazyl (DPPH) was procured from Sigma Chemical Co., St Louis, MO (USA).

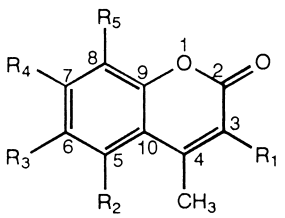
Test compounds (coumarins 1–23)

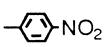
The 4-methylcoumarins **1–4**, **9–16**, **19–21** (Table 1) were synthesised by the well-known Pechmann condensation of the corresponding di/trihydroxyphenol (resorcinol, phloroglucinol, pyrogallol or 1,2,4-trihydroxybenzene) with ethyl acetoacetate or its derivatives having ethoxycarbonylmethyl or ethoxycarbonylethyl substituents at the methylene carbon, followed by methylation and acetylation by standard procedures.[14,15] The physical and spectral data of compounds **1–4**, **9–16** and **19–21** assayed for activity in this communication have been published by us earlier.[13,15–18] The compounds **5–8**, **17**, **18**, **22** and **23** were obtained from the collection of the late Professor T. R. Seshadri, FRS in Delhi.

Preparation of rat liver microsomes

Rat liver microsomes used for lipid peroxidation studies were prepared adopting the method of Ernster and Nordenbrand.[19] Freshly excised rat liver was suspended in 0.25 M sucrose solution and homogenized to obtain 30% homogenate which was centrifuged at 10,000 *g* for 30 min in a Sorvall refrigerated centrifuge. The supernatant was spun at 100,000 *g* for 1 h in Beckman ultracentrifuge (model L) and the surface of pelleted microsomes was rinsed with 0.15 M KCl and resuspended in 0.15 M KCl. Protein was assayed by the method of Lowry et al.[20]

Table 1. Effects of 4-methylcoumarins on NADPH-dependent lipid peroxidation in rat liver microsomes



Compd	R_1	R_2	R_3	R_4	R_5	Percent of control
1	H	OH	H	OH	H	69
2	$CH_2CH_2COOC_2H_5$	OH	H	OH	H	85
3	$CH_2CH_2COOC_2H_5$	H	OH	OH	H	33
4	H	H	H	OH	OH	11
5		H	H	OH	H	98
6		OH	H	H	H	119
7		H	Br	OH	H	92
8	H	H	OH	H	H	102
9	H	$OCOCH_3$	H	$OCOCH_3$	H	24
10	$CH_2COOC_2H_5$	$OCOCH_3$	H	$OCOCH_3$	H	100
11	H	H	$OCOCH_3$	$OCOCH_3$	H	14
12	$CH_2CH_2COOC_2H_5$	H	$OCOCH_3$	$OCOCH_3$	H	20
13	H	H	H	$OCOCH_3$	$OCOCH_3$	13
14	$CH_2CH_2COOC_2H_5$	H	H	$OCOCH_3$	$OCOCH_3$	15
15	$CH_2COOC_2H_5$	H	H	$OCOCH_3$	H	143
16	H	H	H	$OCOCH_3$	H	100
17		H	$OCOCH_3$	H	H	119
18	H	$OCOCH_3$	H	OCH_3	H	95
19	$CH_2COOC_2H_5$	H	H	OCH_3	OCH_3	84
20	H	OCH_3	H	OCH_3	H	141
21	$CH_2CH_2COOC_2H_5$	H	H	OCH_3	H	155
22	H	H	OCH_3	H	H	83
23	H	OCH_3	H	H	H	97

Assay for initiation of lipid peroxidation

The reaction mixture in a final volume of 2 mL consisted of 0.025 M tris–HCI (pH 7.5), microsomes (1 mg protein), 3 mM ADP and 0.15 mM $FeCl_3$. The tubes were pre-incubated for 10 min at 37 °C followed by the addition of the test compounds added at a concentration of 100 μM in 0.2 mL of DMSO and then again incubated for 10 min at 37 °C. The reaction was started by the addition of 0.5 mM NADPH for initiation of enzymatic lipid peroxidation and incubated for different intervals. The reaction was terminated by the addition of 0.2 mL of 50% TCA, followed by addition of 0.2 mL of 5 N HCI and 1.6 mL of 30% TBA. The tubes were heated in an oil bath at 95 °C for 30 min, cooled and centrifuged at 3000 rpm. The intensity of the colour of the thiobarbituric acid reactive substance (TBRS) formed was read at 535 nm. The lipid peroxidation was found to be linear upto 15 min under the conditions described here.

Assay of radical chain reaction using linoleic acid lipid hydroperoxide

Linoleic acid hydroperoxide was prepared essentially as described by Sato et al.[21] The assay mixture consisted of 10 mM phosphate buffered saline containing 140 mM NaCl (pH 7.4), 10 mM linoleic acid (freshly prepared), 50 mM EDTA, 0.1 mM $FeSO_4$ and the test compounds (100 μM). The reaction was started by the addition of linoleic acid hydroperoxide (approximately 15 μM) at 37 °C for different intervals. The reaction was terminated and TBRS measured as described earlier.

Assay of DPPH radical scavenging

A solution of test compounds in methanol (4 mL) at various concentrations ranging from 1 to 400 μM depending on the potency of the inhibitor was added to 1 mL of DPPH solution in methanol (0.15 mM). The contents were vigorously mixed, allowed to stand at 20 °C for 30 min and the absorption was read at 517 nm.

Results

We have in this investigation examined the effect of a wide variety of 4-methylcoumarin derivatives on initiation, propagation and chain termination of lipid peroxidation in rat liver microsomes. The results (Table 1) illustrate the influence of 4-methylcoumarins on the initiation of lipid peroxidation enzymatically. The various substituents on coumarin nucleus are one or two hydroxy, acetoxy and methoxy group(s) alone or in combination with other groups. In addition, the effect of substitution at the C-3 position in the form of an alkyl chain containing 0–5 carbon atoms on initiation of lipid peroxidation has been investigated. The monomethoxycoumarins **22** and **23** were found to cause very little inhibition of lipid peroxidation. Similarly dimethoxycoumarin **19** and monohydroxycoumarins **7** and **8** were found ineffective to inhibit microsomal lipid peroxidation. However, the monohydroxy-, monomethoxy- and dimethoxycoumarins **6**, **20** and **21** enhanced the lipid peroxidation depending on the position of other substituents. Contrary to the effect of aforementioned functionalities, the dihydroxy and diacetoxy system produced dramatic inhibition of lipid peroxidation in rat liver microsomes. 7,8-Dihydroxy-4-methylcoumarin (**4**) inhibited microsomal lipid peroxidation to the extent of 90% (Table 1), intiated enzymatically by the addition of NADPH to the reaction mixture. The relative position of hydroxy groups in the benzenoid ring of the coumarin demonstrated the selectivity for inhibiting the lipid peroxidation. The relative antioxidant activities exhibited by the dihydroxycoumarins **1–4** examined in this study have been found to be dependent on the relative positions of the two hydroxy groups, the order being: 7,9-dihydroxy > 6,7-dihydroxy > 5,7-dihydroxy (Table 1). The diacetoxycoumarins **9–14** like the above mentioned dihydroxycoumarins profoundly inihibited the initiation of lipid peroxidation (Table 1). The activity of the diacetoxycoumarins has also been found to be dependent on the relative positions of the two acetoxy groups, the order again being 7,8-diacetoxy > 6,7-diacetoxy > 5,7-diacetoxy (Tables 1 and 2). These observations necessitate location of hydroxy and acetoxy groups to be at the *ortho* positions to each other for maximum inhibitory activity. It is worth noting that hydroxyl or acetoxy groups at the *meta* position in the phenyl ring of coumarin nucleus contribute very little to the inhibition of lipid peroxidation (Table 1). The dihydroxy- and diacetoxycoumarins were screened for their

Table 2. Inhibitory action of 4-methylcoumarins on propagation of lipid peroxidation

Inhibitor	μmol of TBRS/mg protein
—	4.04
7,8-Diacetoxy-4-methylcoumarin (**13**)	0.38
7,8-Diacetoxy-3-ethoxycarbonylethyl-4-methylcoumarin (**14**)	1.36
6,7-Diacetoxy-4-methylcoumarin (**11**)	0.42
6,7-Diacetoxy-3-ethoxycarbonylethyl-4-methylcoumarin (**12**)	1.46
7,8-Dihydroxy-4-methylcoumarin (**4**)	0.73
α-Tocopherol	0.93

$EDTA\text{-}FeSO_4$ was added to a mixture of autoxidized linoleic acid and linoleic acid hydroperoxide to propagate, the lipid peroxidation process as described under Materials and Methods. The inhibitor concentration was 100 μM. Values represent mean of three separate experiments with variation of < 5%.

ability to inhibit propagation of lipid peroxidation by subjecting linoleic acid hydroperoxide to undergo further peroxidation by the action of EDTA–$FeSO_4$ added to the reaction mixture. The results in Table 2 highlight the remarkable effectiveness of dihydroxy- and diacetoxycoumarins in inhibiting the propagation of lipid peroxidation. It is interesting to note that 7,8-diacetoxy-4-methylcoumarin surpasses α-tocopherol in effecting the inhibition of linoleic acid-induced lipid peroxidation (Table 2). Similarly 7,8-dihydroxy-4-methylcoumarin was also found to be a profound inhibitor (Table 2). The relative inhibitory potential of dihydroxy and diacetoxy derivatives of 4-methylcoumarin are highlighted in Table 3. Remarkably 7,8-diacetoxy-4-methylcoumarin is nearly 126 times more potent than α-tocopherol in forging the inhibition of initiation of lipid peroxidation in microsomal membrane. Efforts were made to examine whether the dihydroxy- and diacetoxycoumarins described above had any ability to terminate the lipid peroxidation. The compounds were added to the reaction mixture 15 and 30 min after the initiation of lipid peroxidation, Figure 1 amply demonstrates the superb antioxidant activity of diacetoxy and dihydroxy derivatives of 4-methylcoumarins to scavenge peroxy radicals involved in lipid peroxidation. The radical scavenging potencies of dihydroxy- and diacetoxycoumarins are documented in Table 4, these are based on the determination of drop in the absorption of the stable radical DPPH. The dihydroxy- and diacetoxycoumarins scavenged DPPH effectively.

Several acetoxybenzenes were examined to see whether they had any antioxidant effect. The results tabulated in Table 5 clearly indicate that they have no antioxidant property compared to coumarin derivatives, thus showing that the biological activity is the combined effect of coumarin nucleus and the *ortho* dihydroxy or diacetoxy system in the benzenoid moiety.

Table 3. Comparison of inhibitory potential of 4-methylcoumarins and α-tocopherol

Inhibitor	IC_{50} (μM)	Intensity of green complex formed
7,8-Dihydroxy-4-methylcoumarin (**4**)	7.90	+ + +
6,7-Diacetoxy-4-methylcoumarin (**11**)	0.69	+ +
6,7-Diacetoxy-3-ethoxycarbonylethy-4-methylcoumarin (**12**)	46.0	+
7,8-Diacetoxy-4-methylcoumarin (**13**)	0.25	+ + + +
7,8-Diacetoxy-3-ethoxycarbonylethyl-4-methylcoumarin (**14**)	0.69	+ + + +
α-Tocopherol	31.62	Nil

The initiation of lipid peroxidation as described under Materials and Methods was followed. The effect of inhibitor concentration ranging from 0.01 μM to 100 μM on initial rate of lipid peroxidation was determined to calculate the inhibitor concentration for 50% inhibition (IC_{50}). The values represent the mean of four experiments with variation of < 5%.

Discussion

Peroxidation of lipids of biomembranes is a complicated process involving formation and propagation of lipid

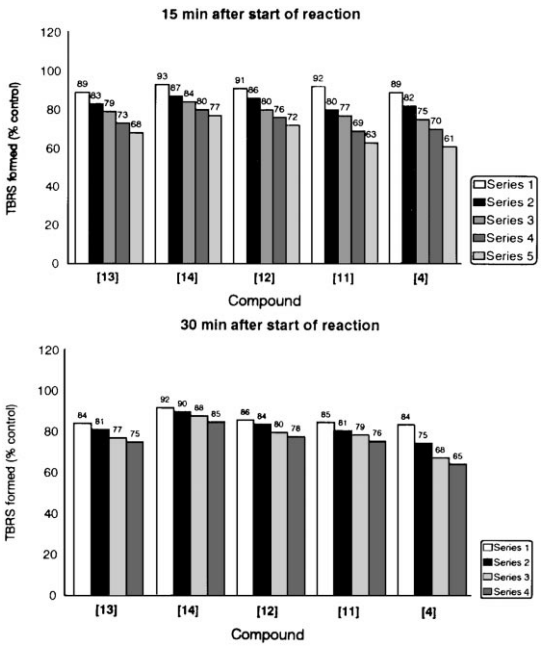


Figure 1. The initiation of lipid peroxidation was carried out as described in Materials and methods. The test compounds were added seperately at a final concentration of 100 μM at 15 and 30 min after the initiation of lipid peroxidation and allowed to proceed for different intervals and TBRS determined. Each histogram represents the % of control at 5 min interval in succession (series 1–4) after the addition of the test compound. Values represent the mean of two different experiments.

Table 4. Radical scavenging potential of diacetoxy and dihydroxy 4-methylcoumarins

Compd	Scavenging of DPPH IC_{50} (μM)
7,8-Dihydroxy-4-methylcoumarin (**4**)	14
6,7-Diacetoxy-4-methylcournarin (**11**)	31
6,7-Diacetoxy-3-ethoxycarbonylethyl-4-methylcoumarin (**12**)	172
7,8-Diacetoxy-4-methylcoumarin (**13**)	31
7,8-Diacetoxy-3-ethoxycarbonylethyl-4-methylcoumarin (**14**)	85
Ascorbic acid	35

The decolourizition of DPPH by various test compounds was carried out as described in Materials and Methods.

Table 5. Effect of acetoxybenzenes on initiation of lipid peroxidation

Test compd	TBRS formed (μmol/mg protein)*
None	6.35
Acetoxybenzene	6.35
1,2-Diacetoxybenzene	6.23
1,3,5-Triacetoxybenzene	6.97
1,2,3-Triacetoxybenzene	6.58

*Values expressed are mean of three separate experiments with variation of $< 5\%$. The details are as described in Materials and methods.

radicals, oxygen uptake and rearrangement of double bonds in unsaturated lipids. Uncontrolled lipid peroxidation ultimately leads to deterioration of membrane lipids yielding a range of degradation products.[22,23] Since microsomal membranes contain high amounts of unsaturated fatty acids, they undergo peroxidation readily in the presence of NADPH, ferric ions and metal chelators such as ADP (Ernster and Nordenbrand[19]). The investigations from the Laboratory of Aust[24] demonstrated that NADPH-dependant lipid peroxidation proceeds through the formation of lipid hydroperoxides called 'initiation step'. This is followed by

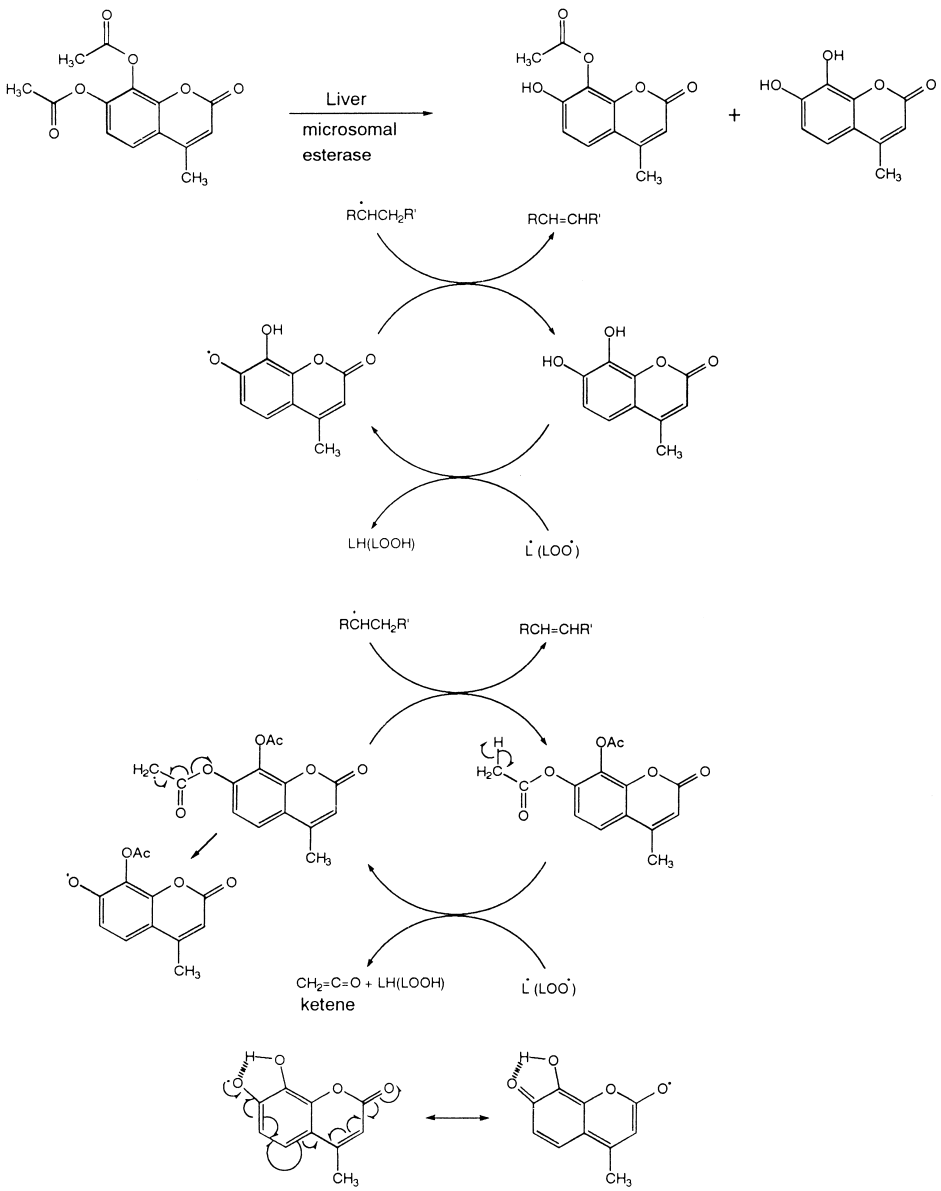


Figure 2. Suggested reaction of DAMC and DHMC showing radical structures.

'propagation' which involves the breakdown of hydroperoxides formed during initiation yielding reactive radicals and products unique to lipid peroxidation.

We have in this investigation utilised the initiation and propagation steps of microsomal lipid peroxidation in order to examine the antioxidant properties of a large number of 4-methylcoumarins. The inhibitory effects of monohydroxy- or monoacetoxycoumarin were very marginal, while there was quantum jump in the potency of dihydroxy and diacetoxy derivatives to retard the initiation of lipid peroxidation. These compounds could abolish nearly 90% of lipid peroxidation. In addition, dihydroxy- and diacetoxycoumarins were found to possess the ability to terminate the radical chain reaction and propagation of lipid peroxidation along with excellent radical scavenging potency. These properties of the aforementioned coumarin derivatives highlight them as superb antioxidants. It is evident from the results that dihydroxy and diacetoxy groups at the position *ortho* to each other impart extraordinary antioxidant activity. The fact that 7,8-dihydroxy- and 7,8-diacetoxy-4-methylcoumarin (DAMC and DHMC, respectively) possess maximum inhibitory effect has been established from the current study (Table 4). Investigations on several types of flavonoids brought out the fact that the catechot moiety in their structure contributes to the antioxidant activity.[25–27] The results obtained in the present study appear to endorse the conclusions drawn from the aforementioned studies on flavonoids.[27] The coumarins examined in the present study also work in the same way and break the chain of lipid peroxidation as shown in Figure 2.

The excellent radical scavenging ability of the dihydroxy derivatives of 4-methylcoumarins may be explained by the fact that the *ortho* dihydroxy system is able to form a resonance stable radical as shown in Figure 2, thus making it feasible for such compounds to scavenge a radical. Such type of resonance stabilised radicals in differently substituted navonoids are known to confer radical scavenging properties to flavonoids.[28] Liver microsomes are known to contain highly active deacetylase catalysing the hydrolysis of a wide variety of endogenous as well as xenobiotics.[29] Microsome-mediated conversion of DAMC to DHMC (Figure 2) has been demonstrated by us (unpublished observation). it is conceivable that DHMC so formed from DAMC enter the radical scavenging pathway (Figure 2).

It is noteworthy that diacetoxy 4-methylcoumarins demonstrate antioxidant activity by ways of radical chain termination of membrane lipid peroxidation (Figure 2) and scavenging of the stable radical DPPH (Table 4) independent of the action of microsomal enzyme. We believe that there exists the possibility of the conversion of DAMC to DHMC in presence of the initiating free radical such as DPPH or Peroxy radical or Superoxide radical through the formation of reactive ketene (Figure 2). DAMC and DHMC are inhibitors of cytochrome P-450 linked mixed function oxidation (unpublished results), a property which may partly contribute to the antioxidant action. It is interesting to note that dihydroxy and diacetoxy derivatives upon addition to the reaction mixture rendered green colour unlike rest of the compounds. Preliminary experiments reveal the formation of a chromophoric complex, studies are underway to explore the role of the green complex in mediating the antioxidant activity.

Acknowledgements

We are grateful to the Danish International Development Agency (DANIDA, Denmark) and the Council for Scientific and Industrial Research (CSIR, New Delhi, India) for financial assistance.

References

1. Scott, G. *Chem Britain* **1995**, 879.

2. Foye, W. O.; Lemke, T. L.; Williams, D. A. (Eds). *Principles of Medicinal Chemistry* (Fourth Edition) BI Waverly Pvt Ltd **1995**, 499.

3. Morel, D. W.; Di Corleto, P. E.; Chisolm, G. M. *Atherosclerosis* **1984**, *4*, 357.

4. Heineke, J. W.; Baker, L.; Rosen, H.; Chait, A. *J. Clin. Invest*. **1986**, *77*, 757.

5. Cody, V.; Middleton, E.; Harborne, J. B. In *Plant Flavonoids in Biology and Medicine*; Alan R Liss: New York, **1986**; Vol. 1, p 15.

6. Middleton, R. H. *Trans. Pharmacol. Sci*. **1984**, *5*, 335.

7. Shimoi, K.; Masuda, S.; Furugori, M.; Esaki, S.; Kinae, N. *Carcinogenesis* **1994**, *15*, 2669.

8. Takeda, S.; Aburada, M. *J. Pharmacobiodyn*. **1981**, *4*, 724.

9. Yang, C. H.; Chiang, C.; Liu, K.; Peng, S.; Wang, R. *Chem. Abstr*. **1981**, *95*, 161758.

10. Tyagi, A.; Dixit, V. P.; Joshi, B. C. *Naturwissenschaftten* **1980**, *67*, 104.

11. Deana, A. A. *J. Med. Chem*. **1983**, *26*, 580.

12. Raj, H. G.; Gupta, S.; Prasad, A. K.; Boll, P. M.; Wengel, J.; Biswas, G.; Singh, S. K.; Sharma, N. K.; Bisht, K. S.; Parmar, V. S. *Bioorg. Med. Chem. Lett*. **1995**, *5*, 1567.

13. Raj, H. G.; Gupta, S.; Biswas, G.; Singh, S.; Singh, A.; Jha, A.; Bisht, K. S.; Sharma, S. K.; Jain, S. C.; Parmar, V. S. *Bioorg. Med. Chem*. **1996**, *4*, 2225.

14. Pechmann, H. V.; Duisberg, C. *Ber*. **1893**, *16*, 2187.

15. Parmar, V. S.; Bisht, K. S.; Jain, R.; Singh, S.; Sharma, S. K.; Gupta, S.; Malhotra, S.; Tyagi, O. D.; Vardhan, A.; Pati, H. N.; Berghe, D. V.; Vlietinck, A. J. *Indian J. Chem*. **1996**, *35B*, 220.

16. Parmar, V. S.; Singh, S.; Rathore, J. S. *J. Indian Chem. Soc*. **1987**, *64*, 254.

17. Parmar, V. S.; Singh, S. *J. Indian Chem. Soc*. **1987**, *64*, 745.

18. Parmar, V. S.; Singh, S.; Rathore, J. S. *J. Chem. Res.* **1984**, 378.

19. Ernster, L.; Nordenbrand, K. *Meth. Enzymol.* **1967**, *10*, 574.

20. Lowry, O. H.; Rosenbrough, W. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.

21. Sato, T.; Kawamoto, A.; Tamura, A.; Tatsumi, Y.; Fuji, T. *Chem. Pharm. Bull.* **1992**, *40*, 721.

22. Gardner, H. W. *J. Agric. Food. Chem.* **1975**, *23*, 129.

23. Gardner, H. W.; Kleiman, R.; Weisleder, D. *Lipids* **1974**, *9*, 696.

24. Svingen, B. A.; Buege, J. A.; O'Neal, F. O.; Aust, S. D. *J. Biol. Chem.* **1979**, *254*, 5899.

25. Husain, S. R.; Cillard, J.; Cillard, P. *Phytochemistry* **1987**, *26*, 2489.

26. Yones, M.; Seigers, C. P. *Planta Medica.* **1981**, *43*, 240.

27. Jha, H. C.; Von Recklinghausen, Zelliken, F. *Biochem. Pharmacol.* **1985**, *34*, 1367.

28. Bors, W.; Heller, W.; Michel, C.; Saran, M. *Meth. Enzymol.* **1990**, *186*, 343.

29. Suzuki-kurasaki, M.; Yoshioko, T.; Uematsu, T. *Biochem. J.* **1997**, *325*, 155.