Pergamon

0031-9422(94)00798-5

Phytochemistry, Vol. 38, No. 5, pp. 1137–1141, 1995

0031-9422/95 $9.50 + 0.00

COMPARATIVE STUDY OF THE INHIBITION OF RAT AND TOBACCO SQUALENE SYNTHASE BY SQUALESTATINS

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(*Received* 29 *July* 1994)

Key Word Index—*Nicotiana tabacum*; Solanaceae; rat liver; squalene synthase (EC 2.5.1.21); inhibition; squalestatins; zaragozic acid.

Abstract—Squalestatins 1–3 and a series of S1 analogues modified at the C-1, C-3, C-4 or C-6 position were able to inhibit squalene synthase, a key enzyme in both cholesterol and phytosterol biosynthesis, in microsomal rich preparations from both rat liver and *N. tabacum*. IC_{50} values varied between 4 and 2000 nM, and similar inhibition values were observed in both systems. The structural requirements for maximal activity at each position are discussed.

INTRODUCTION

Elevated serum cholesterol levels in man have been established as an important factor in the aetiology of atherosclerosis and the application of cholesterol lowering regimens are of proven efficacy in decreasing the incidence of coronary heart disease. The search for agents which may lower serum cholesterol levels has to date, unlike that for anti-steroidogenic fungicides, been concentrated on the pre-squalene span of the pathway [1]. Recent attempts to identify novel, potent and selective inhibitors of mammalian squalene synthase have largely concentrated on the chemical synthesis of putative transition state analogues of the reaction. Examples of these include farnesyl diphosphate analogues [2] and more recently a series of isoprenoid 1,1-bisphosphonates which are effective squalene synthase inhibitors at nanomolar concentrations [3] and have been shown to be effective inhibitors of cholesterol biosynthesis in rats on intravenous and oral dosing [4]. Analogues of presqualene pyrophosphate including presqualene phosphonophosphates [5] and ammonium analogues [6] have also been examined as squalene synthase inhibitors but these exhibited only modest inhibition.

As part of a screening program to seek inhibitors of squalene synthase, Dawson *et al.* [7] found potent activity against both mammalian (rat liver) and fungal (*Candida albicans*) enzymes in the fermentation broth of a newly isolated species of *Phoma* (sp. C2932) (Coelomycetes). Subsequent investigations established that the inhibition was due to a novel family of compounds designated the squalestatins whose structures were determined by mass spectrometry and NMR spectroscopy [8]. The three squalestatins (S1–S3), which represent the first novel class of squalene synthase inhibitors from a natural source, were all potent inhibitors of squalene synthesis from [1-^{14}C]IPP or [2-^{14}C]FPP in a rat liver system with IC_{50} values of between 4 and 22 nM making them approximately 100 times more active than any previously described compounds. Subsequent to these initial publications a group at Merck reported the isolation and structure elucidation of zaragozic acid A which is identical to squalestatin 1(S1) [9, 10].

Investigations into the biosynthesis of the major squalestatin (S1) by means of incorporation studies using a variety of labelled precursors [11] suggested that the main carbon skeleton was built up by polyketide chain extension of the aromatic starter unit benzoic acid, C-methylation, and finally the condensation of the α-methylene group of the chain with an α-keto dicarboxylic acid.

The effectiveness of S1 (and by implication the other squalestatins) in lowering serum cholesterol levels *in vivo* has also been demonstrated in marmosets [12], a species which has a similar lipoprotein profile to that of man, with cholesterol lowering of 75% obtained with oral dosing of 100 mg kg^{-1} day^{-1}.

S1 has also been isolated from *Setosphaeria khartoumensis* by another group and the inhibition of the two partial reactions of squalene synthase investigated [13]. They found that S1 had an IC_{50} of 3 nM for the overall reaction and that the reaction was competitive with respect to FPP with a K_i value of 1.6 nM. The first partial

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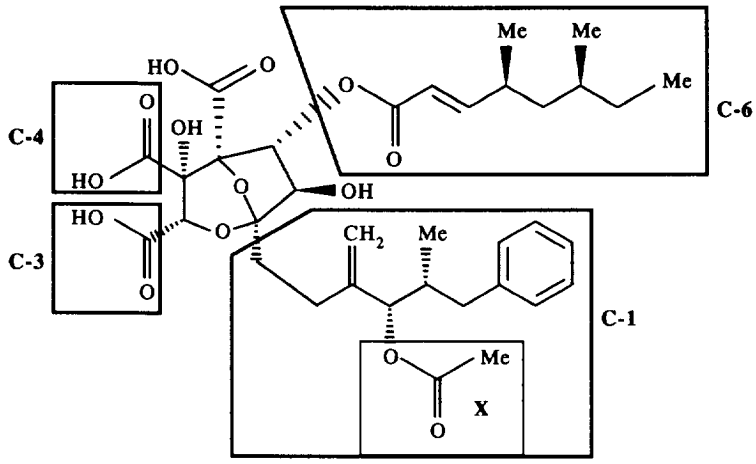
reaction (assayed by release of $^3H^+$ from [1-$^3H_2$]FPP in the absence of NADPH) was inhibited by 50% at 14 nM, the second partial reaction [determined by measuring squalene synthesis from pre-[^{3}H]squalene pyrophosphate (PSPP) in the presence of NADPH] was inhibited by more than 90% at 34 nM.

The detailed mechanism by which squalene synthase is inhibited by S1 is at present unclear. There is some structural similarity between S1 (squalestatin) and PSPP (each compound consists of a central hydrophilic moiety connected to two hydrophobic chains). Procopiou *et al.* [14] have suggested that those squalestatins containing a 4,6-dimethyloctanoate dimethyloctanoate ester at C-6 are PSPP mimetics, whilst those with only an hydroxyl group at C-6 are FPP mimetics.

In this paper we report on the effects of the three squalestatins (S1–S3) originally isolated from *Phoma* by Dawson *et al.* [7] and a series of chemically modified analogues (S4–S13) (Fig. 1), prepared by Glaxo Group Research on squalene synthase activity in a microsomal preparation from rat liver and a microsomal-rich preparation from *N. tabacum*.



Compound	Modification(s)				
	C-1	C-3	C-4	C-6	X
Natural					
Squalestatin 1 (S1)	—	—	—	—	—
Squalestatin 2 (S2)	—	—	—	—	OH
Squalestatin 3 (S3)	—	—	—	OH	—
Analogues					
S4	—	—	—	OH	OH
S5	—	—	CO_2Me	—	—
S6	—	—	—	H	—
S7	—	CO_2Me	—	—	—
S8	—	CH_2OH	—	—	—
S9	—	H	—	—	—
S10	—	CH_2OH	—	—	OH

Fig. 1. Chemical structures of squalestatins 1–3 and their analogues.

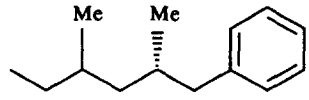
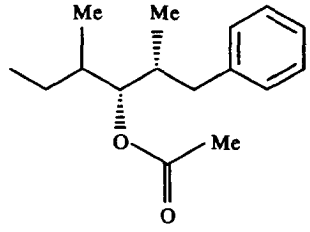
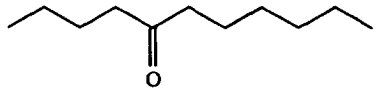
Compound	Modification of S1 at C-1 (all other positions unmodified)
S11	
S12	
S13	

Fig. 1. *Continued.*

RESULTS AND DISCUSSION

Squalene synthase activity was measured by means of a high throughput microtitre assay in which the amount of radioactive squalene formed from [2-^{14}C]FPP is measured after the selective elution from a silica gel TL matrix of the unused [2-^{14}C]FPP present in the assay mixture at the end of the incubation period. The IC_{50} values for each inhibitor were determined from graphs such as that shown for S3 (see Fig. 2). The values obtained using both the rat liver and tobacco preparations were generally of the same order of magnitude for all of the squalestatins tested and varied between 4 and < 1000 nM (Table 1), this is in contrast to the observed differences in sensitivity of some other squalene synthase inhibitors reported for yeast and mammalian enzymes [4].

The most potent squalene synthase inhibitor tested in the experiments reported in this study was squalestatin 2 (S2) which had IC_{50} values of 5 and 4 nM in rat liver and tobacco, respectively. The other two natural squalestatins, S1 and S3, gave IC_{50} values of 100 and 40 nM, respectively and were among the most active tested. Modification of the C-4 position of S1 from $-CO_2H$ to

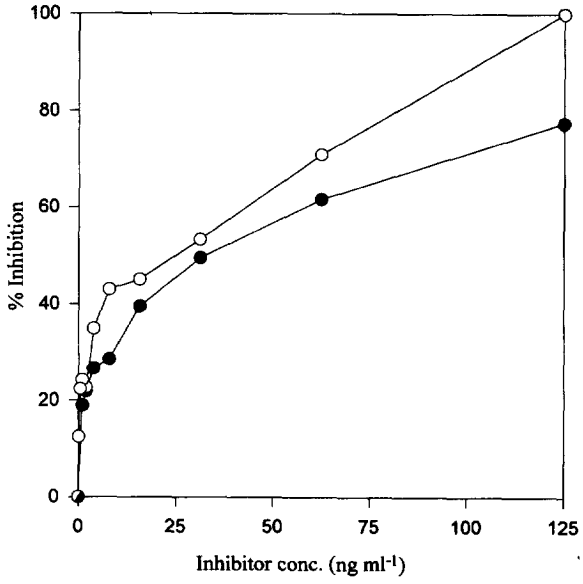


Fig. 2. Inhibition of squalene synthase as a percentage of their respective controls in rat liver (●—●) and *N. tabacum* (○—○) microsomal preparations by squalestatin 3 (S3). The values represent the average of three assays (which varied by less than 5%) for each inhibitor concentration.

Table 1. IC_{50} values for the squalestatins in rat liver and *N. tabacum* microsomal preparations

	IC_{50}(nM)	
Compound	Rat liver	*N. tabacum*
S1	46	100
S2	5	4
S3	60	40
S4	900	2000
S5	60	75
S6	160	200
S7	180	280
S8	14	24
S9	140	150
S10	<700	<700
S11	13	39
S12	400	260
S13	<1000	<1000

IC_{50} values were calculated by means of graphical analysis (see Fig. 2).

$-CO_2Me$ (S5) resulted in no significant loss of activity, while modification of the C-3 position from $-CO_2H$ to $-CH_2OH$ (S8) actually resulted in a small increase of activity to 14 and 24 nM in rat liver and tobacco, respectively. By contrast, modification of the C-3 position of S1 to either $-CO_2Me$ (S7) or $-H$ (S9) resulted in a slight loss in acitivity (~ 200 nM), this result was consistent with previously reported results in rat liver [15]. Interestingly when the C-3 position was modified to $-CH_2OH$ as in S8 (which had high activity) and position X was modified to $-OH$ (as in S10) there was an almost complete loss of activity. The presence of hydroxyl groups at position C-6 and X also resulted in an almost complete loss of activity.

Modification of the whole hydrocarbon chain at C-1 (rather than just the X position) revealed that the O_2Me at position -X could be removed (S11) or the benzene ring replaced by cyclohexane (S12) without significant loss of activity, if the group was replaced with a linear hydrocarbon chain (S13) there was complete loss of activity (again this is consistent with previously reported results for rat liver [15]).

Although the mechanism of action of the squalestatins is at present unknown, a number of structural features required for their activity in both rat liver and tobacco are apparent. Firstly, the C-1 side chain must contain a six-membered ring at its terminal position, although it can be either saturated or unsaturated. Secondly, the C-3 position can contain either $-CO_2Me$, $-CH_2OH$ or H. This situation is complicated by the fact that the combination of groups found at the C-3 and C-4 positions, not just the groups in isolation at these positions, is probably important in determining activity. Thirdly, the presence of a substituent at either the C-6 or the X position is essential for activity.

Analysis of the deduced amino acid sequence of squalene synthase from human, *S. pombe* and *S. cerevisiae*, although demonstrating similarities in overall protein architecture, in particular the conserved arrangement of hydrophillic and hydrophobic domains, established that there is only a 36% conservation of identical residues [15]. This compares to the situation in HMG-CoA reductase, a key regulatory enzyme in sterol biosynthesis, in which there is a conservation of residues of 65% between *S. cerevisiae* and humans [16]. In addition other evidence of evolutionary changes in the above three squalene synthases include the finding of small gaps (2–5 residues) in the amino acid alignment and the absence of a single 28 residue domain in the human enzyme [15]. It seems likely that the differences in sensitivity of several other squalene synthase inhibitors reported previously for yeast and mammalian enzymes is related to these structural differences.

EXPERIMENTAL

Radiochemicals and chemicals. [2-^{14}C]FPP (55 μCi μM^{-1}) was purchased from the Radiochemical Centre, Amersham. Squalestatins were supplied by Glaxo Group Research, Greenford.

Biological material. The growth and maintenance of the *N. tabacum* L. var. White Burley cell suspension cultures and the prepn of a cell-free system have been described previously [18]. Microsomal-rich frs were obtained by centrifugation of a cell-free prepn (120000 *g*, 1 hr). The prepn of the microsomal fr. from rat liver was carried out as described previously [19].

Assay of squalene synthase. This was performed using a high throughput microtitre assay as described previously [19]. The assay is based on the principle of selective elution from a solid phase; briefly the production of radiolabelled squalene from [2-^{14}C]FPP is determined by applying the reaction mixt. to silica gel TLC sheets and then selectively eluting the FPP precursor into a soln of sodium dodecyl sulphate at alkaline pH. In order to prevent further metabolism of the squalene and hence provide a dedicated squalene synthase assay an endogenous oxygen consumption system (ascorbate/ascorbate oxidase) is incorporated into the assay mixt.

The standard assay mixt. (total vol. 50 μl) contained 50 mM MOPS/NaOH, pH 7.5, 10 mM KF, 10 mM $MgCl_2$, 0.5 mM NADPH, 36 μM [2-^{14}C]FPP (0.1 μCi per assay), 50 mM ascorbate, 20 units ml^{-1} ascorbate oxidase, 10 μl of inhibitor prepn and 1–3 mg protein ml^{-1} rat liver microsomal protein or 1–2 mg protein ml^{-1} of *N. tabacum* microsomal-rich fr. Incubations were carried out in microtitre plates (Nunc) for 30 min at room temp. and terminated by the addition of 50 μl propan-2-ol.

The reaction mixt. (25 μl) was applied to polyester-backed silica gel TLC plates (scored into 2 cm squares) using positive displacement pipettes. After gentle drying the plates were washed in three changes (20 min each) of 0.1 M ethanolamine/1% (w/v) SDS, pH 11 (no pH adjustment). The squares were then cut from the plate and placed into a scintillation vials for counting. Controls were carried out to determine the rate of labelled squalene production equivalent to no inhibition each time the assay was performed and inhibition values calculated as a percentage of this value. TLC analysis (hexane–EtOAc, 9:1) of an aliquot of the reaction mixt. confirmed that squalene was the only radiolabelled product of the assay.

Acknowledgements—We wish to thank Dr Melanine O'Neil for help and advice and Glaxo Group Research Ltd for financial support. D.C.F. was in receipt of a Glaxo Research Studentship.

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