DESIGN AND SYNTHESIS OF SQUALENE SYNTHASE INHIBITORS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
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VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN
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ADRIANUS ROBERTUS PETRUS MARIA VALENTIJN

GEBOREN TE TER AAR IN 1965

Samenstelling Promotiecommissie

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STELLINGEN

behorende bij het proefschrift

DESIGN AND SYNTHESIS OF SQUALENE SYNTHASE INHIBITORS

1. Abdel-Rahman *et al.* gaan voorbij aan het feit dat naast D-gulose en D-idose ook L-glucose als uitgangsstof gebruikt kan worden voor de synthese van de bicyclische kern van de zaragozic acids.
Abdel-Rahman, H.; Adams, J.P.; Boyes, A.L.; Kelly, M.J.; Mansfield, D.J.; Procopiou, P.A.; Roberts, S.M.; Slee, D.H.; Watson, N.S. *J. Chem. Soc. Chem. Commun.* **1993**, 1839.

2. Het valt te betreuren dat het succes van de door Wu gepresenteerde synthese en toepassing van allylische barium Grignards, afhankelijk is van een onbekende verontreiniging in een van de gebruikte reagentia.
Wu, T.-C.; Hiong, H.; Rieke, R.D. *J. Org. Chem.* **1990**, *55*, 5045.
Corey, E.J.; Shieh, W.-C. *Tetrahedron Lett.* **1992**, *33*, 6435.

3. De manier waarop Oehlschlager *et al.* de structuur van de door hen gesynthetiseerde squaleen synthase remmers aangeven is verwarrend.
Oehlschlager, A.C.; Singh, S.M.; Sharma, S. *J. Org. Chem.* **1991**, *56*, 3856.

4. Het is verwonderlijk dat Myers *et al.* in hun artikel over de synthese van (+)-Tunicamycine V geen aandacht besteden aan de mogelijke substitutie van het primaire bromide tijdens de reductie van de azide groep met fenylselenol.
Myers, A.G.; Gin, D.Y.; Rogers, D.H. *J. Am. Chem. Soc.* **1993**, *115*, 2036.

5. De solid-phase synthese van oligosaccharides biedt alleen voordelen ten opzichte van de synthese in oplossing als de glycosyleringsreacties stereoselectief en bij kamertemperatuur verlopen.
Danishefsky, S.J.; McClure, K.F.; Randolph, J.T.; Ruggeri, R.B. *Science* **1993**, *260*, 1307.
Yan, L.; Taylor, C.M.; Goodnow Jr., R; Kahne, D. *J. Am. Chem. Soc.* **1994**, *116*, 6953.

6. Overzichtsartikelen waarin de structuurformules van chirale verbindingen onjuist zijn weergegeven hebben een toegevoegde didactische waarde.
Rollin, P.; Klaffke, W. *J. Carbohydrate Chem.* **1991**, *10*, 115.

7. Het gebruik van het voorvoegsel α of β om de configuratie van het anomere centrum in 1,x-anhydro suikers aan te geven is overbodig.

8. Het valt te betwijfelen of het gebruik van loodvrije benzine een bijdrage levert aan een schoner milieu.

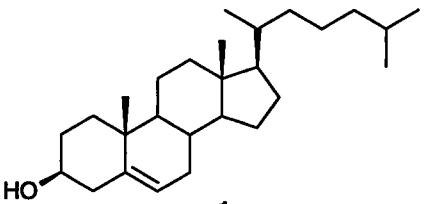
Contents

List of Abbreviations

b	broad
Bn	benzyl
Bu	*n*-butyl
CoA	coenzyme A
d	doublet
DCC	*N,N'*-dicyclohexylcarbodiimide
DMAPP	dimethylallyl pyrophosphate
DMEM	Dulbecco's modified eagles medium
DMF	*N,N*-dimethylformamide
DTT	dithiothreitol
FPP	farnesyl pyrophosphate
FPPA	FPP analogue
GPP	geranyl pyrophosphate
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
IC_{50}	dose that inhibits 50% of the activity of *e.g.* an enzyme
IPP	isopentenyl pyrophosphate
LDL	low density lipoprotein
m	multiplet
MVA	mevalonic acid
NBS	*N*-bromosuccinimide
NCS	*N*-chlorosuccinimide
NMR	nuclear magnetic resonance
PFT	protein:farnesyl transferase
PPi	pyrophosphoric acid
PPP	presqualene pyrophosphate
q	quartet
s	singulet
SEM	standard error of mean
sex	sextet
SQL	squalene
SS	squalene synthase
t	triplet
TEAB	triethylammonium bicarbonate
THF	tetrahydrofuran
TMS	trimethylsilyl
Trt	trityl

General Introduction

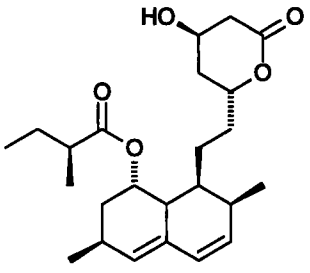
It is widely recognized now that elevated blood plasma cholesterol levels are a high risk factor for cardiovascular diseases, which are still the major death cause in the modern western society. For instance, atherosclerosis is responsible for half of the deaths in the



1

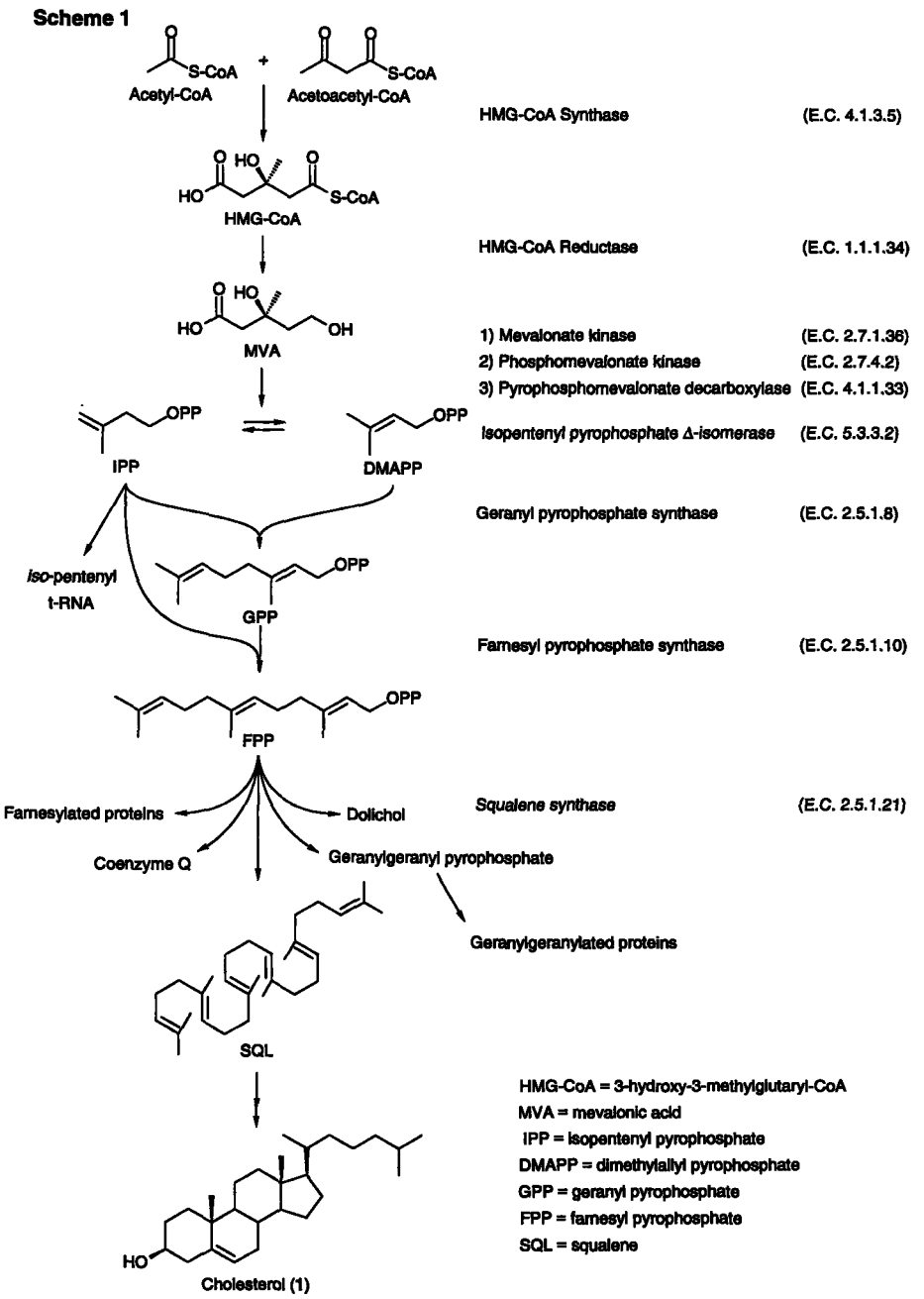
United States.[1,2a] A characteristic feature of this disease is the accumulation of cholesterol (**1**) in the walls of arteries, eventually resulting in obstruction of the blood-vessel leading to a stroke or heart attack.[1] The cholesterol in these atherosclerotic plaques is mainly derived from cholesterol esters in Low Density Lipoproteins (LDL), a particle in the blood which is responsible for transport of cholesterol through the body. Cholesterol enters the body by absorption from the diet (300-500 mg per day) or results from endogenous synthesis (700-900 mg per day).[3] The main site of biosynthesis is the liver, which processes acetyl-CoA to cholesterol, as depicted in Scheme 1.[4]

An important approach towards lowering blood plasma cholesterol levels entails inhibition of cholesterogenesis. Several enzymes[2] in the biosynthetic route to cholesterol have been the target of inhibition. A major class of compounds comprises HMG-CoA reductase inhibitors,[5] which obstruct the formation of mevalonic acid (MVA) from



2

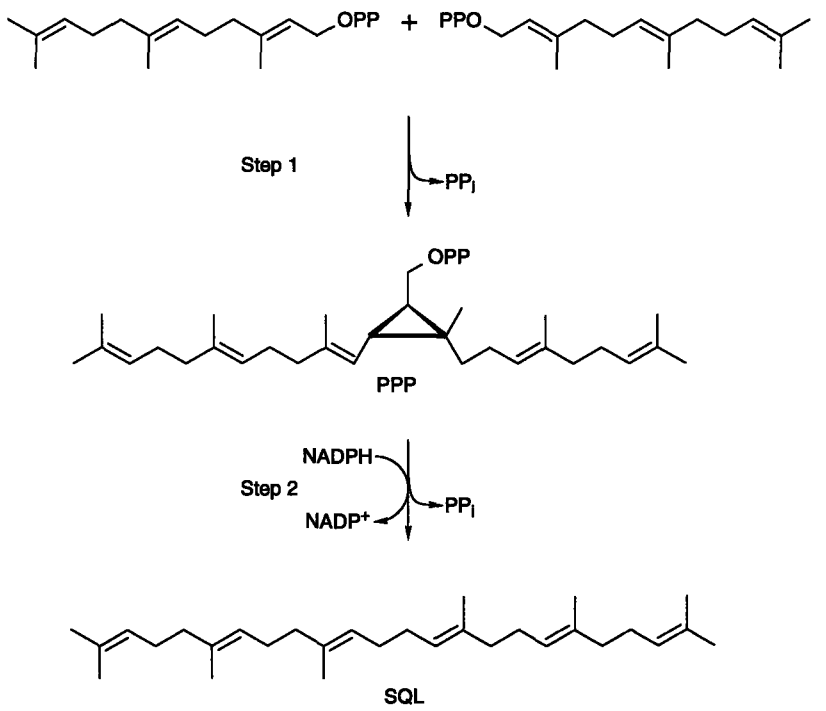
Scheme 1


Acetyl-CoA
Acetoacetyl-CoA
HMG-CoA Synthase (E.C. 4.1.3.5)
HMG-CoA
HMG-CoA Reductase (E.C. 1.1.1.34)
MVA
1) Mevalonate kinase (E.C. 2.7.1.36)
2) Phosphomevalonate kinase (E.C. 2.7.4.2)
3) Pyrophosphomevalonate decarboxylase (E.C. 4.1.1.33)
IPP
DMAPP
Isopentenyl pyrophosphate Δ-isomerase (E.C. 5.3.3.2)
Geranyl pyrophosphate synthase (E.C. 2.5.1.8)
iso-pentenyl t-RNA
GPP
Farnesyl pyrophosphate synthase (E.C. 2.5.1.10)
FPP
Farnesylated proteins
Dolichol
Squalene synthase (E.C. 2.5.1.21)
Coenzyme Q
Geranylgeranyl pyrophosphate
Geranylgeranylated proteins
SQL
Cholesterol (1)
HMG-CoA = 3-hydroxy-3-methylglutaryl-CoA
MVA = mevalonic acid
IPP = isopentenyl pyrophosphate
DMAPP = dimethylallyl pyrophosphate
GPP = geranyl pyrophosphate
FPP = farnesyl pyrophosphate
SQL = squalene

3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), the rate-limiting step in the biosynthesis of cholesterol. For instance, lovastatin (2) is an effective therapeutic agent for the lowering of both total cholesterol and LDL-cholesterol (30-50%) in patients with primary hypercholesterolemia at doses of 20-80 mg per day.[5] One major disadvantage of inhibiting HMG-CoA reductase is the fact that the enzyme occupies an early position in the terpene biosynthetic pathway. Thus, apart from inhibiting the cholesterol biosynthesis, the formation of important side-products such as isopentenyl t-RNA,[6] isoprenylated proteins,[7] coenzyme Q[8] and dolichol[9] may also be influenced.

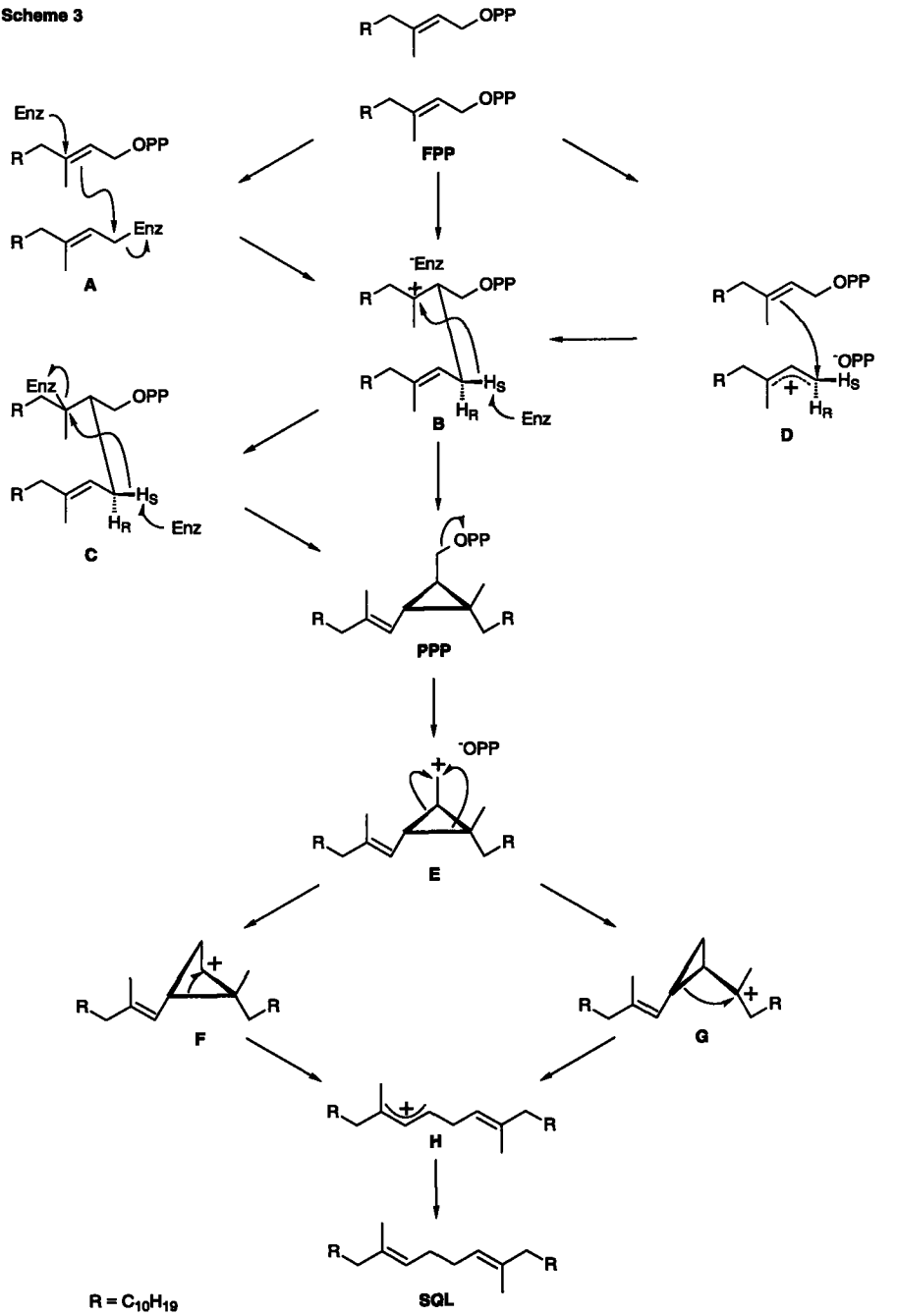
To overcome this drawback, attention was shifted to squalene synthase (SS), a 47 kDa enzyme that is localized in the membrane of the endoplasmic reticulum of mammalian

Scheme 2



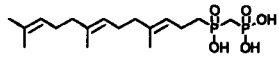
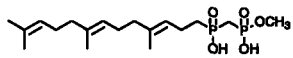
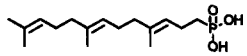
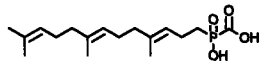
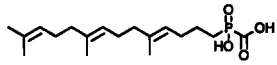
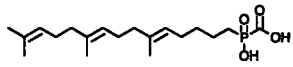
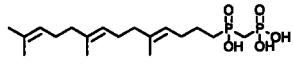
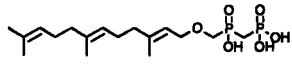
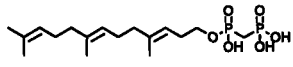
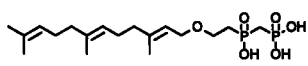
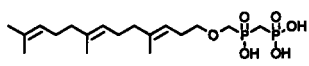
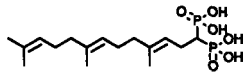
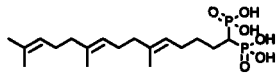
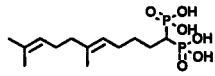
liver and yeast,[10-13] at the final branch-point of the cholesterol biosynthetic pathway. SS catalyses, as delineated in Scheme 2, the reductive dimerization of two molecules farnesyl pyrophosphate (FPP) to give squalene (SQL). It was shown by Rilling[14] in 1966 that the enzymic head-to-head condensation of two FPP-units proceeded in two distinct steps. However, it was not until 1969[15] that presqualene pyrophosphate (PPP) was shown to be the intermediate of the reaction. Since that time, different mechanisms[10,16-26] (see Scheme 3) have been suggested for the two separate steps. In the first step, two FPP molecules are

Scheme 3



asymmetrically combined to give the cyclopropyl containing PPP and inorganic pyrophosphate. For instance, Beytia *et al.*[20] proposed that a ping-pong reaction was involved in the formation of PPP, according to the sequence **FPP→A→B→C→PPP**. Recently, Mookhtiar *et al.*[16] proposed that the formation of a tight ion-pair of an allylic cation and inorganic pyrophosphate (**D**) is followed by an insertion reaction of the cation in the C2-C3 double bond of the second FPP (**D→B→PPP**) to give PPP. The mechanism of the second step, the conversion of PPP to SQL is believed to proceed *via* a cyclopropylcarbinyl cation

Table 1. Representative examples of FPP-analogues

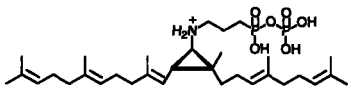
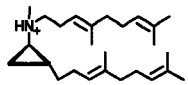
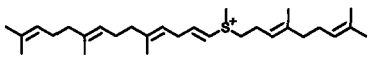
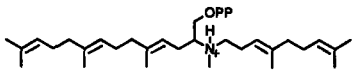
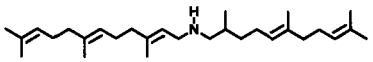
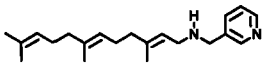
Entry	Compound	IC_{50} (μM)	Ref.
1		31.5	35
2		177	35
3		N.I.[a]	35
4		100	31
5		31.8	31
6		>300	31
7		67	32
8		0.05	32
9		16	32
10		2.3	32
11		0.15	32
12		0.032	27
13		0.0057	27
14		0.00095	27

[a]N.I. = Non Inhibitory

(E), followed by rearrangement to a cyclobutyl (F) or cyclopropyl (G) intermediate and a second rearrangement to the squalene cation (H), which is subsequently reduced with NADPH to SQL. Unfortunately, none of these mechanisms could be completely verified by mechanistic studies.

Through the years, many inhibitors of SS have been described in the literature.[26-40] It is of interest to note that analogues based on geranyl pyrophosphate (GPP) do not inhibit SS, since GPP is no substrate for the reaction. A major class of compounds comprises FPP-analogues,[27,28,31,32,34,35,38,40] most of which contain a modified pyrophosphate function. Inhibitory studies of these analogues on SS revealed some interesting aspects. For instance, initially it was shown[35] that at least three negative charges had to be present in the polar head of the molecule (entries 1-3 in Table 1). In contrast, the phosphinylformates[31] showed a remarkable inhibitory capacity with only two acid groups present (entries 4-6). Furthermore, the latter group of compounds exhibit an optimal inhibitory action when the farnesyl residues and the polar head of the molecule are separated by two atoms. The first potent inhibitor of SS was reported by Biller *et al.*[32] and contains an oxy-methylphosphinylmethylphosphonate unit (entry 8). This analogue is approximately 1000 times more active than its corresponding carbon isostere (entry 7). On the basis of this finding they concluded that the allylic oxygen plays an important role in

Table 2. Representative examples of SS-inhibitors based on putative cationic intermediates

Entry	Compound	Based on*	Ref.
1		E	36
2		G	36
3		H	33
4		B	30
5		H	29
6		H	29

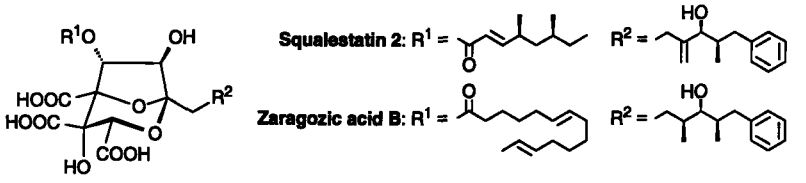
*See Scheme 2

binding to SS. In addition it was demonstrated that an optimal inhibitory action is attained when the oxygen and the α-phosphorus in these molecules are separated by one carbon atom (entries 8-11). An even more potent class of compounds was very recently presented by Ciosek *et al.*.[27] The latter inhibitors support the mechanism of the conversion of FPP into PPP as proposed by Mookhtiar *et al.*[16] (**FPP→D→PPP** in Scheme 3), *i.e.* the bisphosphonic acid inhibitors in entries 12-14 are supposed to mimic the tight ion-pair of inorganic pyrophosphate and the allyl cation.

Apart from the inhibitors based on FPP, several research groups pursued the synthesis of analogues[29,30,33,36,37,39,40] (some representative examples are recorded in Table 2) of putative cationic intermediates in the enzymic formation of SQL. However, the inhibitory potency of these ammonium[30,36] (entries 1,2 and 4) and sulfonium[33] (entry 3) ion analogues was rather low in comparison with the inhibitors recorded in Table 1. On the other hand, Prashad *et al.*[29] recently described a series of *N*-(arylalkyl)farnesyl-amines (see for instance entries 5 and 6), some of which exhibited the same degree of SS-inhibition as the FPP-analogues in entries 8-11 of Table 1.

Surprisingly, the most potent and selective inhibitors of SS were not designed and synthesized on the basis of proposed mechanisms of the conversion of FPP into SQL, but were obtained by screening of fungal metabolites. This resulted in the discovery of the squalestatins[41-44] and zaragozic acids[45-48] (see for example squalestatin 2 and zaragozic acid

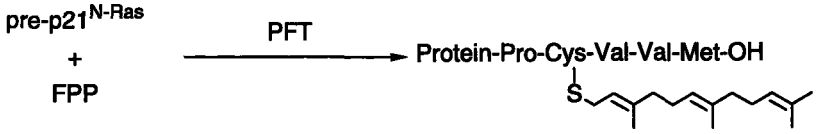
Figure 1



B in Fig. 1), isolated from the cultures *Phoma* sp. C2932, *Sporormiella intermedia* and *Leptodontium elatius*. Structure elucidation of this new class of compounds revealed that the common element in the squalestatins and zaragozic acids is the (1S-(1α,3α,4β,5α,6α,7β))-4,6,7-trihydroxy-2,8-dioxabicyclo[3.2.1]octane-3,4,5-tricarboxylic acid core, whereas they are distinguished from each other by the presence of different acyl and alkyl side-chains at O6 and C1. Dufresne *et al.*[47] postulated that the inhibitory action of the squalestatins (zaragozic acids) on SS may be explained by their topological similarity with PPP, *i.e.* both structures of the squalestatins and PPP are characterized by the presence of rigid triacid cores flanked by two lipophilic residues.

As mentioned earlier (see Scheme 1), FPP may also function as a substrate in the biosynthesis of farnesylated proteins, coenzyme Q, geranylgeranyl pyrophosphate and

Scheme 4



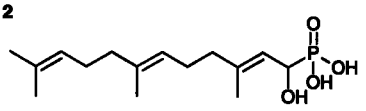
dolichol. Recently, much attention has been focused on the farnesylation of proteins,[7] a post-translational modification, catalyzed by the enzyme protein:farnesyl transferase (PFT). The latter enzyme covalently links a farnesyl residue to cysteine at the C-terminal region of certain proteins belonging to the family of small G-proteins (see *inter alia* the farnesylation of human pre-$p21^{N\text{-}Ras}$ in Scheme 4). PFT selectively recognizes the so-called CAAX-box of pre-Ras,[49] a C-terminal sequence of four amino acids in which C stands for cysteine, A may be any aliphatic amino acid and X serine, methionine or phenylalanine. After attachment of the farnesyl group the protein is further processed by proteolytic removal of the AAX amino acid residues. Finally, methyl esterification of the carboxyl of cysteine engenders the active Ras protein.

The farnesylated Ras proteins are involved in the signalling pathways[50] used by cells to respond to growth factors. The farnesyl residue serves as an anchor to facilitate membrane association of Ras by lipophilic interactions. Alternatively, the farnesyl moiety may be recognized by a membrane-bound receptor and thus be localized in the cell membrane. It has been shown that mutated Ras, which is constantly 'turned on', in combination with other regulatory abnormalities is responsible for uncontrolled cell division.[50] The latter is endorsed by the fact that mutated Ras is found in human colon cancers (approximately 50%)[51] and pancreatic carcinomas (nearly 90%).[51]

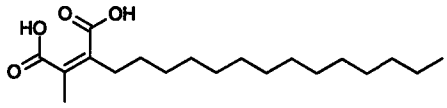
Since farnesylation of (mutated) Ras is essential for its function, inhibition of PFT is an attractive target to block uncontrolled cell division. Several approaches[52] to inhibit Ras farnesylation have been described in the literature. An important strategy is based on the fact that PFT selectively recognizes the CAAX-box of pre-Ras. Thus, CAAX-mimics may act as inhibitors of PFT. Indeed, it was shown[53-58] that tetrapeptide analogues of CAAX are competitive inhibitors of PFT. In addition, James *et al.*[50] demonstrated that benzodiazepine peptidomimetics are also capable of preventing farnesylation of Ras.

Several classes of natural products[59-61] were identified as inhibitors of PFT. For instance, Hara *et al.*[60] performed microbial screening and isolated a new culture of *Streptomyces*, producing potent PFT-inhibitors, designated UCF1 (see UCF1 A-C in Fig. 2). Furthermore, chaetomellic acids A and B,[61,62] isolated from *Chaetomella acutiseta*, block Ras processing *in vivo* by selective inhibition of PFT. Molecular modelling studies indicated that the inhibitory action of these acids could be explained by their structural resemblance with FPP.

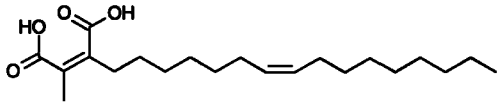
Figure 2



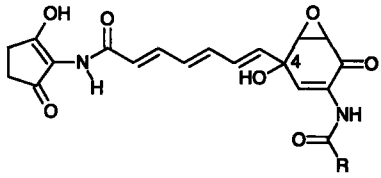
α-Hydroxyfarnesylphosphonic acid



Chaetomellic acid A



Chaetomellic acid B



UCF1-A (4-R): R =

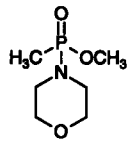
UCF1-B (4-S): R =

UCF1-C (4-R): R =

Recently, Pompliano *et al.*[63] presented evidence that the FPP-analogue α-hydroxyfarnesyl phosphonate (see Fig. 2) is efficient in preventing Ras farnesylation. From the latter finding it may be concluded that FPP-analogues have not only the potency to inhibit SS, but also PFT.

In order to get a better insight in the structural requirements of SS- and PFT-inhibitors we set up a program to design and synthesize several compounds which may function as inhibitor of SS or PFT.

In *Chapter 1* the synthesis of the new phosphonylating agent methyl methylphosphonomorpholidate (**3**) is described. The versatility of reagent **3** is demonstrated in the preparation of FPP-analogues **4**-**6**. We show that compound **3** can be employed as a



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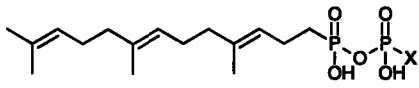
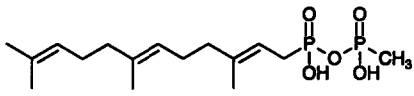


4: X = OH

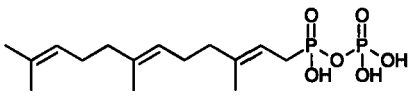
5: X = CH_3



6

building block for the α-phosphorus in compounds **4** and **5**, whereas it is also suitable for the introduction of the β-phosphorus in compound **6**.

Chapter 2 describes a convenient method for the preparation of phosphonophosphate and phosphonophosphonate analogues of FPP (**6-9**). In the classical approach towards this type of modified pyrophosphates, DCC and morpholine are used to convert a phosphonic acid into the corresponding phosphonomorpholidate. The latter is condensed *in situ* with phosphoric acid to yield a phosphonophosphate. However, this methodology proved to be unsatisfactory in a synthetic approach to compounds **8** and **9**. The construction of the latter



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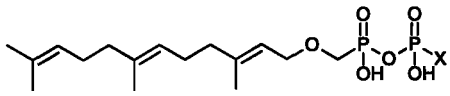
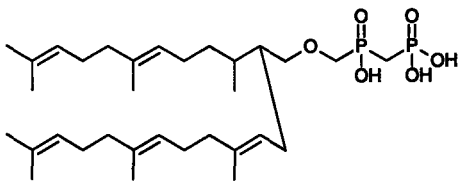


8: X = OH
9: X = CH_3

compounds could be achieved by transforming the phosphonic diester into the corresponding mono-chloride and subsequent reaction with morpholine to give the phosphonomorpholidate ester. Removal of the remaining ethyl-ester function followed by coupling with phosphoric or methylphosphonic acid gave the target compounds.

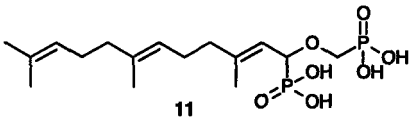
The inhibitory action of compounds **4-9** in a SS- and PFT-assay is the subject of the *Addendum*. This study describes the structural requirements of the modified pyrophosphate function for binding to either SS or PFT.



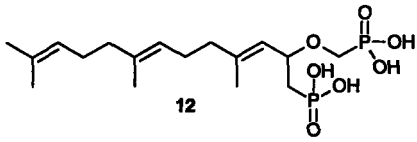
10

The synthesis of compound **10** is described in *Chapter 3*. Compound **10** is considered to be the analogue of putative intermediate **C** in Scheme 3. Furthermore, evidence is presented for the inhibitory action of **10** on SS.

In *Chapter 4* attention is focused on the synthesis of compounds **11-15** containing two phosphonic acid moieties. Compounds **11** and **12** were readily accessible by reaction of farnesal with diethyl phosphite or dimethyl lithiomethylphosphonate, respectively, followed by condensation of the resulting alcohols with diethyl phosphonomethyl triflate. The preparation of **13** and **14** was accomplished by alkylation of bis(diethyl phosphono-methyl) ether or tetraethyl methylenebisphosphonate with farnesyl bromide. Further



11



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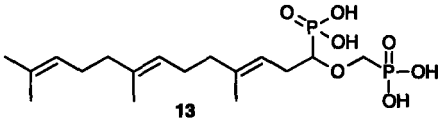

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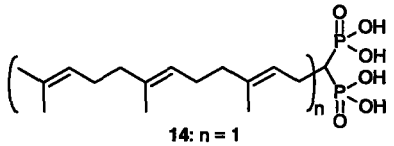
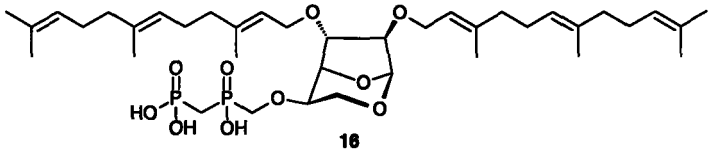


14: n = 1
15: n = 2

extension of **14** with a farnesyl residue gave the bisfarnesylated product **15**.

The synthesis of 1,6-Anhydro-2,3-di-*O*-farnesyl-5-*O*-([{phosphonomethyl}-phosphinyl]methyl)-α-D-galactofuranose **16** is described in *Chapter 5*. We show that this hybrid of zaragozic acid and PPP can be prepared in 12 steps in an overall yield of 1% starting from D-galactose diethyl dithioacetal.



16

In conclusion, this Thesis describes the design and synthesis of potential SS- and PFT-inhibitors using readily available starting materials. Moreover, two new approaches towards the synthesis of modified pyrophosphates are described. The results of the enzyme-assays may pave the way to the design and synthesis of more effective and selective inhibitors of SS and PFT.

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CHAPTER 1

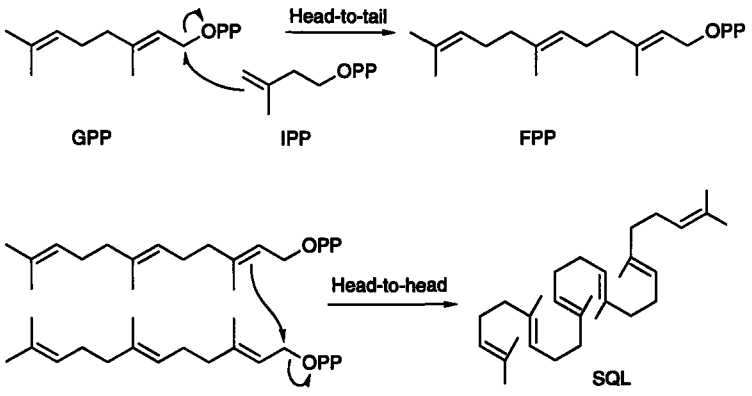
An Expeditious Synthesis of Pyrophosphate Analogues of Farnesyl Pyrophosphate Using the Phosphonylating Agent Methyl Methylphosphonomorpholidate[1]

Abstract: Reaction of methyl lithiomethylphosphonomorpholidate with farnesyl chloride followed by demethylation and subsequent coupling with phosphoric acid or methylphosphonic acid gave farnesylmethylphosphonophosphate (**7**) and farnesylmethylphosphonomethylphosphonate (**8**), respectively. Furthermore, conversion of **2** into methylphosphonomorpholidate (**9**) followed by coupling with farnesylphosphonic acid (**10**) gave farnesylphosphonomethylphosphonate (**11**).

Introduction

The formation of new carbon-carbon bonds is a key process in the multistep biosynthetic pathway to terpenoids[2]. For example, the first step in the enzymic head-to-tail carbon coupling of geranyl pyrophosphate (GPP) with isopentenyl pyrophosphate (IPP) entails (Scheme 1) an intermolecular nucleophilic attack of the double bond in IPP on the allylic GPP carbon atom. The initial enzymatic process results in the release of a pyrophosphate anion and the formation of an intermediate cationic species which, after

Scheme 1



stereospecific elimination of a proton at C-2, engenders farnesyl pyrophosphate (FPP). Apart from this, head-to-head joining of two FPP units proceeds in a similar fashion to afford eventually squalene (SQL), which will be further metabolized via SQL 2,3-epoxide to cholesterol.

Some time ago, Corey[3] reported *inter alia* the synthesis of a C-substituted methylphosphonophosphate analogue (*i.e.* **7**) of FPP, and presented evidence that this methylene analogue inhibited SQL biosynthesis.

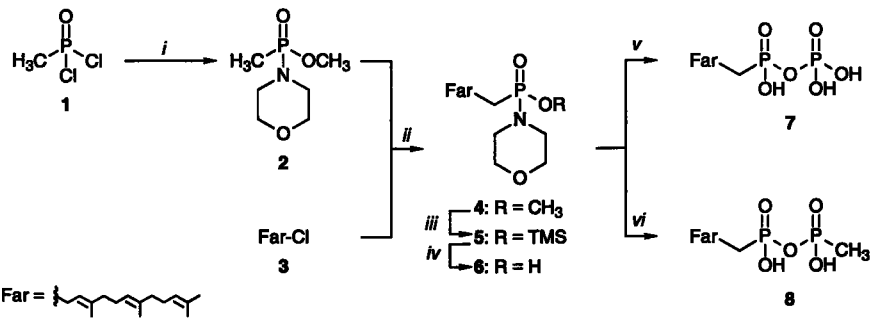
As part of a program directed towards the design and synthesis of potential inhibitors of cholesterol biosynthesis, we here report that the new trifunctional agent methyl methylphosphonomorpholidate (**2**) gives an easy access to C- and P-substituted analogues (*i.e.* **7**, **8**, and **11**, respectively) of FPP.

Results and discussion

The phosphonylating agent **2** could be readily prepared by the two step procedure illustrated in Scheme 2. Treatment of commercially available methylphosphonic dichloride **1** with morpholine followed by the addition of methanol and triethylamine gave, after distillation, homogeneous **2** (δ_P 32.8 ppm) in 79% yield.

The versatility of **2** was firstly demonstrated in the preparation of the two C-substituted analogues **7** and **8** via precursor **6**. Thus treatment of farnesyl chloride (**3**)[4] with methyl lithiomethylphosphonomorpholidate in dry tetrahydrofuran, obtained *in situ* by reaction of 1 equiv *n*-butyllithium with **2** at -78°C, gave, after work up and purification by flash chromatography, homogeneous **4** (δ_P 33.0 ppm) as a colourless oil. Transformation

Scheme 2[a]

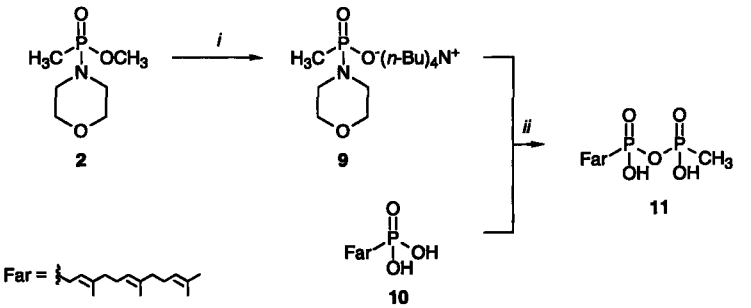


[a]Reagents and conditions

i: 1) Morpholine, ether, 0°C, 2) methanol, triethylamine, ether (79%); *ii*: *n*-BuLi, THF, -78°C (63%); *iii*: TMS-Br, CH_3CN; *iv*: $(n\text{-}Bu)_4NF$, dioxane; *v*: H_3PO_4, pyridine (65%); *vi*: $CH_3H_2PO_3$, pyridine (69%).

of **4** into **6** could be easily effected via the following two-step hydrolysis procedure. Reaction of **4** in acetonitrile with trimethylsilyl bromide[5] (1.5 equiv) for 1 h at 20°C showed, as gauged by ^{31}P NMR spectroscopy, complete conversion of **4** into the silyl ester **5** (δ_P 27.9 ppm), which was isolated in a sufficient pure state after evaporation of excess reactant and solvent. Complete removal of the TMS-group from **5** proceeded smoothly (1h at 20°C) with fluoride ion [$(n\text{-Bu})_4NF$; 1 equiv] in dry dioxane to furnish **6** (δ_P 20.0 ppm), which was used without further purification in the next steps. For instance, condensation of **6** with phosphoric acid (tri-*n*-butylammonium salt; 3 equiv) in dry pyridine (2 mL) for 48 h at 20°C gave, after work up and purification, homogeneous **7**, the ^{31}P NMR spectrum of which showed the characteristic pattern (*i.e.* dd, $J_{P\text{-}P}$ = 26.9 Hz) of the phosphonophosphate function. In a similar fashion, analogue **8** was obtained by treating **6** with methylphosphonic acid.

Scheme 3[a]



[a]Reagents and conditions

i: 1) TMS-Br, CH_3CN, 2) $(n\text{-Bu})_4NF$, dioxane; *ii*: pyridine (70%).

The scope of the phosphonylating agent **2** was further illustrated (Scheme 3) by the synthesis of farnesylphosphonomethylphosphonate **11**. Thus, two-stage hydrolysis of **2**, as mentioned earlier for the de-esterification of **4** → **6**, gave methyl phosphonomorpholidate **9** (δ_P 26.8 ppm) in a nearly quantitative yield. Coupling of the latter, under the same conditions as applied for the synthesis of the analogue **7**, with the recently reported[6] farnesylphosphonic acid **10** gave homogeneous **11**.

In conclusion, the presence in the readily accessible agent **2** of three potentially active centres, which can be manipulated individually, promises to be of great value for the effective introduction of modified pyrophosphate functions. The latter aspect is nicely examplified by the fact that the morpholido function in **2** circumvents the thus far used[3,6] rather cumbersome and timeconsuming transformation of a farnesylphosphonic acid into the corresponding morpholidate with DCC and morpholine[7].

Experimental

General procedures

(E,E)-Farnesol and methylphosphonic dichloride were purchased from Aldrich and distilled. Pyridine was dried by refluxing with CaH_2 for 16 h and then distilled, redistilled from *p*-toluenesulfonyl chloride (60 g/L), redistilled from KOH (40 g/L) and stored over molecular sieves (0.4 nm). Toluene, dichloromethane and ether were dried by refluxing with P_2O_5 for 2 h and then distilled. Toluene and ether were stored over sodium wire. Dichloromethane was stored over molecular sieves (0.4 nm). THF, dioxane, acetonitrile and triethylamine were dried by refluxing with CaH_2 for 16 h and then distilled. Dioxane was stored over molecular sieves (0.5 nm). THF, acetonitrile and triethylamine were stored over molecular sieves (0.4 nm). THF and ether were redistilled from $LiAlH_4$ directly before use. Methanol was dried by refluxing with magnesium methoxide, distilled and stored over molecular sieves (0.5 nm). TLC-analysis was performed on silicagel (Schleicher & Schull, F 1500 LS 254). Compounds were visualised by spraying the TLC-plates with $KMnO_4$ (1%) in aqueous Na_2CO_3 (2%). Column chromatography was performed on Merkc Kieselgel (230-400 Mesh ASTM). Evaporations were carried out below 40°C under reduced pressure (15 mm Hg). ^1H, ^{13}C and ^{31}P NMR spectra were measured at 199.99, 50.1 and 80.7 MHz, respectively, using a JEOL JNM-FX 200 spectrometer on line with a JEC 980 B computer. ^1H, ^{13}C and ^{31}P NMR spectra were recorded using a Bruker WM-300 spectrometer operating at 300, 75 and 121 MHz, respectively. ^1H and ^{13}C chemical shifts are given in ppm (δ) relative to tetramethylsilane (TMS) as internal standard, and ^{31}P chemical shifts are given in ppm (δ) relative to 85% H_3PO_4 as external standard.

Methyl methylphosphonomorpholidate (2)

Methyl phosphonic dichloride (1) (6.65 g, 50 mmol) was dissolved in freshly distilled ether (50 mL) and cooled to 0°C. Then morpholine (8.72 mL, 100 mmol) in ether (20 mL) was added dropwise over a period of 2 h and stirring was continued overnight at 0°C. The salts were removed by filtration, and the solvent was evaporated. The residue was dissolved in ether (50 mL) and treated with a mixture of methanol (3.82 mL, 100 mmol) and triethylamine (6.97 mL, 50 mmol) in ether (50 mL) at 0°C and the reaction mixture was stirred for 24 h at 0°C. The salts were removed by filtration and the filtrate was concentrated at reduced pressure. The oily residue was purified by distillation (bp. 118°C at 0.9 mbar) to give 2 in 79% yield.
$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 7.9, 10.8 (CH_3, $^1J_{C,P}$ = 144.9 Hz); 43.4 (CH_2); 51.6, 51.7 (OCH_3); 66.7 (CH_2).
$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 32.8

(E,E)-Farnesyl chloride (3)

N-Chlorosuccinimide (0.66 g, 4.95 mmol) was dissolved in dichloromethane (20 mL) under a nitrogen atmosphere. The solution was cooled to -30°C and dimethylsulfide (0.37 mL, 5 mmol) was added. The mixture was allowed to warm to 0°C before it was cooled to -40°C. Then farnesol (1 g, 4.5 mmol) dissolved in dichloromethane (2.5 mL) was added dropwise over a period of 3 minutes. The reaction mixture was warmed to 0°C in 1 h, at which temperature it was maintained for another hour. After 15 minutes of stirring at room temperature the reaction mixture was poured in a separatory funnel which contained cold saturated NaCl (12.5 mL). The aqueous layer was extracted with two portions of pentane (10 mL). The organic layers were combined with an additional 10 mL of pentane and washed with two portions of cold saturated NaCl (5 mL).

The organic layer was dried over $MgSO_4$ and concentrated to dryness *in vacuo*. Compound **3** was thus obtained in a yield of 89%.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 16.0, 17.6 (C13, C14, C15); 25.7 (C12); 26.1, 26.7, 39.4, 39.7 (C4, C5, C7, C8); 41.1 (C1); 120.3, 124.0, 124.3 (C2, C6, C10); 130.3, 135.5 (C3, C7, C9).

^1H NMR ($CDCl_3$) δ 1.60 (s, 6H, 2xCH_3); 1.68 (s, 3H, CH_3), 1.72 (s, 3H, CH_3); 2.01-2.35 (m, 8H, H_4, H_5, H_8, H_9); 4.07, 4.11 $J_{1,2}$ = 8 Hz (d, 2H, H_1); 7.15-7.29 (m, 3H, H_2, H_6, H_{10})

(E,E)-Methyl farnesylmethylphosphonomorpholidate (4)

Compound **2** (376 mg, 2.1 mmol) was dissolved in freshly distilled THF (10 mL) and cooled to -78°C under an argon atmosphere. Then *n*-BuLi (1.3 mL, 1.6 M in hexane, 2.1 mmol) was added and stirring was continued for a period of 30 minutes, followed by dropwise addition of **3** (481 mg, 2 mmol) in THF (5 mL) over a period of 5 minutes. After additional stirring for 1 h at -78°C the reaction was quenched by addition of saturated NH_4Cl. The organic layer was diluted with ether, washed with H_2O and brine and dried over $MgSO_4$. The volatiles were removed by evaporation and the residue was purified by silica gel chromatography (elution: CH_2Cl_2/acetone 1/0 → 9/1 v/v) to give compound **4** as a colourless oil in a yield of 63%.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.9, 17.5 (C14, C15, C16); 25.6 (C13); 20.7, 20.8 (C2); 23.4, 26.1 (C1); 26.3, 26.6 (C5, C9); 39.4, 39.6 (C5, C10); 52.0, 52.1 (2xOCH_3); 122.6, 122.9 (C3); 123.8, 124.9 (C7, C11); 131.0, 134.9, 136.4 (C4, C8, C12).

^1H NMR ($CDCl_3$) δ 1.58 (s, 6 H, 2xCH_3), 1.62 (s, 3 H, CH_3), 1.68 (s, 3 H, CH_3); 1.71-1.82 (m, 2 H, H1); 1.97-2.09 (m, 8 H, H5, H6, H9, H10); 2.23-2.34 (m, 2 H, H2); 3.72, 3.76 (2xs, 6 H, 2xOCH_3); 5.07-5.30 (m, 3 H, H3, H7, H12).

$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 33.0.

(E,E)-Farnesylmethylphosphonomorpholidate (6)

Compound **4** (383 mg, 1 mmol) was evaporated three times with toluene and dissolved in acetonitrile (2 mL). Then trimethylsilyl bromide was added (145 µL, 1.1 mmol). As ^{31}P NMR (δ 27.9) showed that the reaction had gone to completion the reaction mixture was concentrated and treated with a 1M solution of TBAF in dry dioxane (1 mL). After 10 min ^{31}P NMR showed that the reaction had gone complete (δ 20.0), thus obtaining compound **6** which was not isolated, but used directly in the next step.

(E,E)-Farnesylmethylphosphonophosphate (7).

Compound **6** was dissolved in pyridine (3 mL) and phosphoric acid (294 mg, 3 mmol, mono tributylammonium salt) was added. Following evaporation of the solvent, the mixture was redissolved in pyridine (2 mL) and stirred at room temperature for 48 h. Then the reaction mixture was concentrated to dryness, dissolved in *iso*-propanol/0.15 M NH_4HCO_3 (1/49 v/v) and applied to a Dowex 50W X 4 cation exchange resin (NH_4^+ form). After elution with the same solvent the eluate was concentrated to dryness. Purification was accomplished by gelfiltration on an S-100 column, eluting with a solution of 30% methanol in 0.15 M NH_4HCO_3. After lyophilization of the appropriate fractions compound **6** was obtained as a white powder in a yield of 65%.

^1H NMR (CD_3OD) δ 1.59 (s, 6 H, 2xCH_3); 1.65, 1.66 (2xs, 6 H, 2xCH_3); 1.99-2.16 (m, 10 H, H1, H5, H6, H9, H10); 2.28-2.39 (m, 2 H, H2); 5.17-5.29 (m, 3 H, H3, H7, H12).

$^{31}P\{^{1}H\}$ NMR (D_2O) δ -9.7, 16.6 (2xd, $J_{P,P}$ = 26.3 Hz)

(E,E)-Farnesylmethylphosphonomethylphosphonate (8)

Compound **6** was dissolved in pyridine (3 mL) and methylphosphonic acid (288 mg, 3 mmol, mono tributylammonium salt) was added. Following evaporation of the solvent, the mixture was redissolved in pyridine (2 mL) and stirred at room temperature for 48 h. Then the reaction mixture was concentrated to dryness, dissolved in *iso*-propanol/0.15 M NH_4HCO_3 (1/49 v/v) and applied to a Dowex 50W X 4 cation exchange resin (NH_4^+ form). After elution with the same solvent the eluate was concentrated to dryness. Purification was accomplished by gelfiltration on an S-100 column, eluting with a solution of 30% acetonitril in 0.15 M NH_4HCO_3. After lyophilization of the appropriate fractions compound **8** was obtained as a white powder in a yield of 69%.

^{1}H NMR (CD_3OD) δ 1.42 (3 H, d, $J_{P,H}$ = 17.22 Hz, CH_3P); 1.60 (s, 6 H, 2xCH_3); 1.64, 1.68 (2xs, 6 H, 2xCH_3); 1.93-2.11 (m, 10 H, H1, H5, H6, H9, H10); 2.18-2.39 (m, 2 H, H2); 5.01-5.28 (m, 3 H, H3, H7, H12).

$^{31}P\{^{1}H\}$ NMR (CD_3OD) δ 17.8, 20.0 (2xd, $J_{P,P}$ = 26.9 Hz)

Methylphosphonomorpholidate (9)

Methyl methylphosphonomorpholidate (**2**) (340 g, 2 mmol) was evaporated three times with toluene and dissolved in acetonitrile (2 mL). Then trimethylsilyl bromide (145 µL, 1.1 mmol) was added. As ^{31}P NMR (δ 24.6) showed that the reaction had gone to completion the reaction mixture was concentrated and treated with a 1M solution of TBAF in dry dioxane (1 mL). Again ^{31}P NMR showed that the reaction had gone complete (δ 20.0), thus obtaining compound **9** which was not isolated, but used directly in the next step.

(E,E)-Farnesylphosphonic acid (10)

To a suspension of sodium hydride (48 mg, 2 mmol) in dry ether (5 mL) was added diethylphosphite (204 mg, 1.5 mmol). After stirring for 2 h a solution of farnesyl chloride **3** (360 mg, 1.5 mmol) in ether (1 mL) was added dropwise and stirring was continued for another 2 h. When TLC analysis (5% acetone in dichloromethane) showed that no starting material was left the solids were removed by filtration. The filtrate was concentrated to dryness and the residue was dissolved in dichloromethane and washed with water, saturated $NaHCO_3$ and water. The organic layer was dried over $MgSO_4$ and evaporated. Chromatographic purification over silica gel (elution: CH_2Cl_2/acetone 1/0 → 9/1 v/v) yielded 83% of diethyl farnesylphosphonate as a colourless oil.

$^{13}C\{^{1}H\}$ NMR ($CDCl_3$) δ15.6, 15.9, 16.1, 16.2 (C13, C14, C15, OCH_2CH_3); 25.3 (C12); 24.6, 27.4 (C1 $J_{P,C}$ = 140.7 Hz); 26.0, 26.1 (C4 $J_{P,C}$ = 2.9 Hz); 26.4 (C8); 39.4 (C5, C9); 61.3, 61.4 (OCH_2CH_3); 112.0, 112.2 (C2 $J_{P,C}$ = 11.7 Hz); 123.5, 124.0 (C6, C10); 130.8, 134.8 (C7, C11); 139.7, 140.0 (C3 $J_{P,C}$ = 14.7 Hz).

$^{31}P\{^{1}H\}$ NMR ($CDCl_3$) δ 28.6.

(E,E)-Diethyl farnesylphosphonate (1 mmol) was evaporated three times with toluene and dissolved in dry acetonitrile (2 mL). Then trimethylsilyl bromide (264 µL, 2 mmol) was added. As ^{31}P NMR (δ 12.1) showed that the reaction had gone to completion the reaction mixture was concentrated and dissolved in a mixture of

20% water in methanol. Again ^{31}P NMR showed that the reaction had gone complete (δ 26.8), thus obtaining compound **10** which was not isolated, but used directly in the next step.

(E,E)-Farnesylphosphonomethylphosphonate (ammonium salt) (11)

Compound **10** was evaporated three times with pyridine and dissolved in pyridine (3 mL). Then a solution of crude **9** in pyridine (3 mL) was added, and the mixture was stirred for 48 h at room temperature. As ^{31}P NMR showed complete conversion of **10** to **11** the reaction mixture was concentrated to dryness, dissolved in *iso*-propanol/0.15 M NH_4HCO_3 (1/49 v/v) and applied to a Dowex 50W X 4 cation exchange resin (NH_4^+ form). After elution with the same solvent the eluate was concentrated to dryness. Purification was accomplished by gelfiltration on an S-100 column, eluting with a solution of 30% methanol in 0.15 M NH_4HCO_3. After lyophilization of the appropriate fractions compound **11** was obtained as a white powder in a yield of 70%. ^1H NMR (CD_3OD) δ 1.42 (d, 3 H, $J_{P,H}$ = 17.22 Hz, CH_3P); 1.60 (s, 6 H, 2xCH_3); 1.67 (s, 6 H, 2xCH_3); 1.85-2.18 (m, 8 H, H4, H5, H8, H9); 2.43-2.61 (m, 2 H, H1); 5.01-5.22, 5.31-5.43 (m, 3 H, H2, H6, H10). $^{31}P\{^1H\}$ NMR (CD_3OD) δ 17.0 (2xd, $J_{P,P}$ = 29.3 Hz)

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CHAPTER 2

A Novel Approach Towards the Synthesis of Pyrophosphate Analogues of Farnesyl Pyrophosphate[1]

Abstract: The synthesis of two new analogues (*i.e.* **3** and **4**) of farnesyl pyrophosphate (FPP) containing a phosphonophosphate and a phosphonophosphonate moiety was accomplished *via* the phosphonomorpholidate. The latter was easily accessible by treatment of a phosphonic chloride with morpholine.

Introduction

Farnesyl pyrophosphate (FPP) occupies an important branch-position in the terpene biosynthetic pathway. Thus, FPP serves as a substrate in the biosynthesis of cholesterol, farnesylated proteins,[2] coenzyme Q,[3] geranylgeranyl pyrophosphate and dolichol.[4] In this complex biosynthetic pathway, squalene synthase (SS) is the first committed enzyme in the biosynthesis of cholesterol and catalyses as depicted in Scheme 1 the head-to-head condensation of two FPP-units to give squalene (SQL). In order to decrease cholesterol biosynthesis, several research groups[5-11] pursued the design and synthesis of SS-inhibitors. For example, Biller *et al.*[9] reported for the first time that the FPP-analogue **1** (see Figure 1) is a potent inhibitor of SS and concluded that the presence of the allylic oxygen in **1** is an important structural feature for binding to SS. This assumption was endorsed by the observation[12] that **1** inhibits SS more effectively than **2**. However, it is not excluded that

Scheme 1

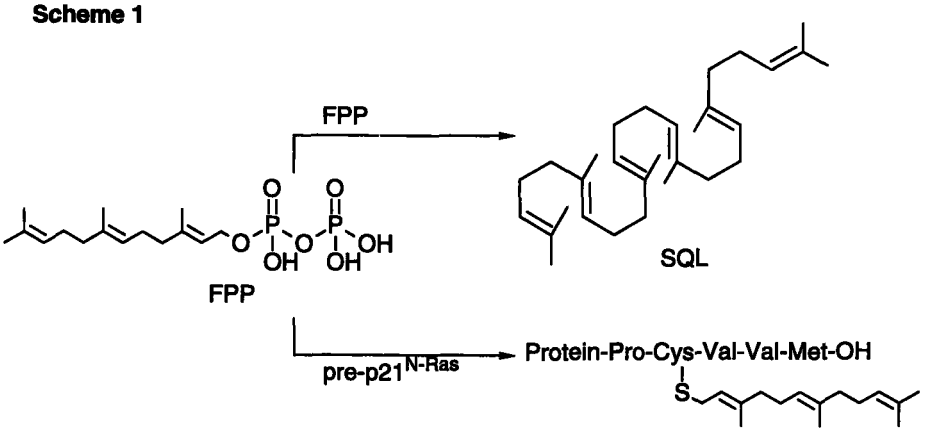
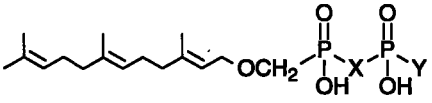
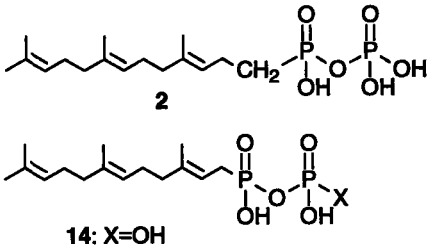


Figure 1



1: X=CH_2, Y=OH
3: X=O, Y=OH
4: X=O, Y=CH_3



2

14: X=OH
15: X=CH_3

FPP-analogues **1** and **2** may also function as inhibitors of other FPP-consuming enzymes. Fortunately, the availability of the enzyme protein:farnesyl transferase (PFT), which is *inter alia* responsible for the farnesylation of human pre-$p21^{N\text{-}Ras}$ at cysteine in the C-terminal region (see Scheme 1), enabled us to explore the inhibitory effect of **1** and **2** in a PFT-assay. Interestingly, the results of the PFT-assay showed[12] that **2** is a more potent inhibitor of PFT than **1**. The inverted inhibition pattern observed in the PFT-assay implies that the oxygen between the two phosphorous atoms of FPP-analogue **2** plays a crucial role in binding to PFT.

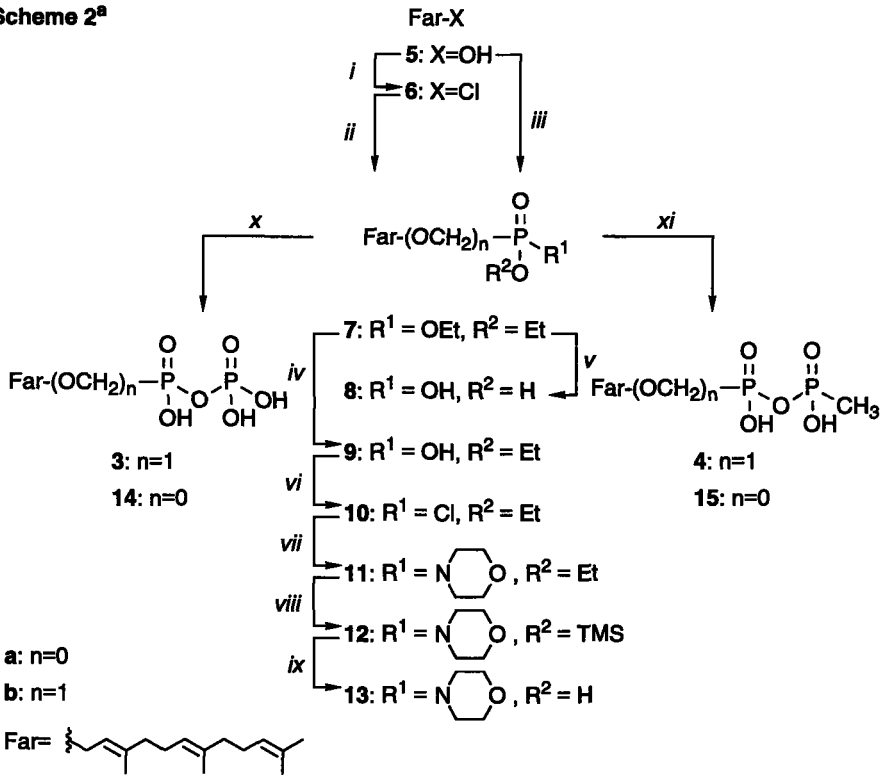
In order to enhance the inhibitory effect of FPP-analogues in the PFT- and/or SS-assay, we here describe the synthesis of **3** and **4** in which a phosphonophosphate or a phosphonophosphonate replaces the phosphinylphosphonate moiety in analogue **1**.

Results and discussion

The crucial step in the synthesis route to potential inhibitors **3** and **4** is the introduction of the respective phosphonophosphate and phosphonophosphonate moieties. In the well established method of Moffatt *et al.*[13] *N,N'*-dicyclohexylcarbodiimide (DCC) and morpholine are used to transform a phosphonic acid into the corresponding mono-morpholidate. The latter is converted *in situ* to the modified pyrophosphate upon treatment with phosphoric acid. Unfortunately, conversion of phosphonic acid **8b** into phosphonomorpholidate **13b** (see Scheme 2) with DCC/morpholine was abortive. In a previous report from this laboratory[10] we showed that methyl methylphosphono-morpholidate[14] is a convenient reagent to introduce the α- and β-phosphonate functions present in FPP-analogues **2** and **15**, respectively. However, any attempt to prepare target compound **4** containing a β-phosphonate moiety failed. Apart from this, it is obvious that target compound **3** is not accessible by the methyl methylphosphonomorpholidate approach. In order to attain our goal we devised an alternative methodology, the key element of which comprises the introduction of the phosphono-morpholidate *via* a phosphonic chloride.

Initially, the viability of this method was explored in the synthesis (see Scheme 2) of the known[8,10] compounds **14** and **15**. To this end, farnesyl chloride (**6**), readily accessible[15] by reaction of farnesol (**5**) with *N*-chlorosuccinimide in the presence of dimethyl sulfide, was treated with the sodium salt of diethyl phosphite generated *in situ* by reaction of sodium with diethyl phosphite. Saponification of the resulting farnesylphosphonate **7a** gave monoester **9a** which was converted into phosphonomorpholidate **11a** by the following one-pot three-step procedure. Silylation of **9a** with *N,N*-diethyl(trimethylsilyl)amine in dichloromethane was followed by addition of oxalyl chloride in the presence of a catalytic amount of *N,N*-dimethylformamide.[16] The crude

Scheme 2[a]



[a]Reagents and conditions

i: *N*-chloro succinimide, dimethyl sulfide, CH_2Cl_2, -40 to 0°C; *ii*: $NaP(O)(OEt)_2$, ether, 0°C; *iii*: 1) *n*-BuLi, THF, -78°C, 2) $TfOCH_2P(O)(OEt)_2$, THF, -78 to 0°C; *iv*: N KOH/ethanol reflux; *v*: 1) TMS-Br, 2) H_2O; *vi*: 1) *N,N*-Et_2NTMS, CH_2Cl_2, 2) oxalyl chloride, DMF, CH_2Cl_2, 0°C; *vii*: morpholine, THF, 0°C; *viii*: TMS-Br, CH_3CN; *ix*: $(n\text{-}Bu)_4NF$, THF; *x*: H_3PO_4, pyridine; *xi*: $CH_3H_2PO_3$, pyridine.

phosphonic chloride **10a** thus obtained, was treated with morpholine in tetrahydrofuran at 0°C, to furnish, after work up and purification, phosphonomorpholidate **11a** in 86% yield. Deblocking of ethyl ester **11a** to give **13a** could be easily effected via the following two-step hydrolysis procedure. Thus, transesterification of **11a** with trimethylsilyl bromide at room temperature led to the TMS-ester **12a**, as gauged by ^{31}P-NMR spectroscopy. Removal of the TMS-group of **12a** with fluoride ion yielded the key phosphonomorpholidate **13a**, which was used without further purification in the synthesis of compounds **14** and **15**. Thus, condensation of **13a** with the tri-*n*-butylammonium salt of phosphoric acid in pyridine for 48 h afforded homogeneous **14** in 70% yield. In a similar way, treatment of **13a** with methylphosphonic acid gave **15** in 69% yield. The physical data of **14** and **15** were in complete accordance with those of the same compounds prepared according to reported procedures.[8,10]

Encouraged by the foregoing results, the same methodology was adopted for the preparation of compounds **3** and **4**. In the first step to common morpholidate **13b**, the triflate group in diethyl phosphonomethyltriflate[17] was replaced with lithium farnesylate to give, as expected,[9] the requisite oxy-methylphosphonate **7b**. Subjection of **7b** to the same protocol as mentioned earlier for the conversion of **7a** into **11a** proceeded smoothly, to yield the morpholidate **11b**. Further, silylation of **11b** and subsequent desilylation of **12b** with tetra-*n*-butylammonium fluoride gave exclusively **13b**, as evidenced by ^{31}P-NMR spectroscopy. Finally, coupling of crude **13b** with phosphoric acid or methylphosphonic acid in pyridine for 48 h resulted in the isolation of **3** and **4** in 50% overall yield.

In conclusion, the results presented in this paper indicate that the introduction of the morpholido function *via* a phosphonic chloride is a versatile and effective approach towards the synthesis of phosphonomorpholidates **13** *en route* to modified pyrophosphates. Furthermore, the intermediate phosphonomorpholidate ethyl esters **11** are shelf-stable and are readily converted into key intermediate **13** by a simple one-pot two-step procedure.

Experimental

General procedures

(E,E)-Farnesol was purchased from Aldrich and distilled. Pyridine was dried by refluxing with CaH_2 for 16 h and then distilled, redistilled from *p*-toluenesulfonyl chloride (60 g/l), redistilled from KOH (40 g/l) and stored over molecular sieves (0.4 nm). Toluene, dichloromethane and ether were dried by refluxing with P_2O_5 for 2 hours and then distilled. Toluene and ether were stored over sodium wire. Dichloromethane was stored over molecular sieves (0.4 nm). THF and acetonitrile were dried by refluxing with CaH_2 for 16 h, distilled and stored over molecular sieves (0.4 nm). THF and ether were redistilled from $LiAlH_4$ directly before use. All reactions were carried out under a blanket of argon, unless stated otherwise. TLC-analysis was performed

on silicagel (Schleicher & Schull, F 1500 LS 254). Compounds were visualised by spraying the TLC-plates with $KMnO_4$ (1%) in aqueous Na_2CO_3 (2%). Column chromatography was performed on Merck Kieselgel (230-400 Mesh ASTM). Evaporations were carried out below 40°C under reduced pressure (15 mm Hg). ^1H, ^{13}C and ^{31}P NMR spectra were measured at 199.99, 50.1 and 80.7 MHz, respectively, using a JEOL JNM-FX 200 spectrometer on line with a JEC 980 B computer. ^1H, ^{13}C and ^{31}P NMR spectra were recorded using a Bruker WM-300 spectrometer operating at 300, 75 and 121 MHz, respectively. ^1H and ^{13}C chemical shifts are given in ppm (δ) relative to tetramethylsilane (TMS) as internal standard and ^{31}P chemical shifts are given in ppm (δ) relative to 85% H_3PO_4 as external standard.

(E,E)-Farnesyl chloride (6)

N-chlorosuccinimide (0.66 g, 4.95 mmol) was dissolved in of dry CH_2Cl_2 (20 mL) under a nitrogen atmosphere. The solution was cooled to -30°C and dimethylsulfide (0.37 mL, 5 mmol) was added. The mixture was allowed to warm to 0°C before it was cooled to -40°C. Then (E,E)-farnesol (1) (1 g, 4.5 mmol) dissolved in CH_2Cl_2 (2.5 mL) was added dropwise over a period of 3 minutes. The reaction mixture was warmed to 0°C in 1 h, at which temperature it was maintained for another hour. After 15 minutes of stirring at room temperature the reaction mixture was poured in a separatory funnel which contained cold saturated NaCl (12.5 mL). The aqueous layer was extracted with two portions of pentane (10 mL). The organic layers were combined with an additional 10 mL of pentane and washed with two portions of cold saturated NaCl (5 mL). The organic layer was dried over $MgSO_4$ and concentrated to dryness *in vacuo* to give farnesyl chloride in a yield of 89%.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 16.0, 17.6 (C13, C14, C15); 25.7 (C12); 26.1, 26.7 (C5, C9); 39.4, 39.7 (C4, C8); 41.1 (C1); 120.3, 124.0, 124.3 (C2, C6, C10); 130.3, 135.5, 142.2 (C3, C7, C11).

^1H NMR ($CDCl_3$) δ 1.60 (s, 6 H, 2xCH_3); 1.68 (s, 3 H, CH_3), 1.72 (s, 3 H, CH_3); 2.01-2.35 (m, 8 H, H4, H5, H8, H9); 4.10 (d, 2 H, H1, $J_{1,2}$ = 7.97 Hz); 4.90-5.21 (m, 2 H, H6, H10); 5.44 (t, 1 H, H2, $J_{2,1}$ = 7.96 Hz).

(E,E)-Diethyl farnesylphosphonate (7a)

Diethyl phosphite (1 mL, 7.75 mmol) was added dropwise to a cooled (0°C) suspension of sodium (172 mg, 7.5 mmol) in ether (10 mL). After stirring for 4 h (E,E)-farnesyl chloride (1.2 g, 5 mmol) was added and stirring was continued at ambient temperature. When TLC-analysis (light petroleum/ether 1/1 v/v) showed that no more starting material was present, the reaction was quenched with ethanol and washed with 25% NH_4Cl, water, dried over $MgSO_4$ and evaporated. Purification of the crude oil by silica gel chromatography (elution: light petroleum/ether 1/0 → 1/1 v/v) gave pure (E,E)-diethyl farnesylphosphonate in 84% yield.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.6, 15.9, 16.1, 16.2 (C13, C14, C15, OCH_2CH_3 Et); 25.3 (C12); 24.6, 27.4 (C1 $J_{P,C}$ = 140.7 Hz); 26.0, 26.1 (C4 $J_{P,C}$ = 2.9 Hz); 26.4 (C8); 39.4 (C5, C9); 61.3, 61.4 (OCH_2CH_3); 112.0, 112.2 (C2 $J_{P,C}$ = 11.7 Hz); 123.5, 124.0 (C6, C10); 130.8, 134.8 (C7, C11); 139.7, 140.0 (C3 $J_{P,C}$ = 14.7 Hz).

^1H NMR ($CDCl_3$) δ 1.32 (t, 6 H; OCH_2CH_3 Et); 1.60, 1.68 (2xs, 12 H, 4xCH_3); 1.88-2.19 (m, 8 H, H4, H5, H8, H9); 2.56 (dd, 2 H, H1, $J_{1,2}$= 7.3 Hz, $J_{H,P}$ = 22.4 Hz); 4.09 (q, 4 H, OCH_2CH_3); 5.04-5.23 (m, 3 H, H2, H6, H10).

$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 28.6.

(E,E)-Diethyl farnesyl-oxy-methylphosphonate (7b)

(E,E)-Farnesol (1.3 mL, 5 mmol) was dissolved in THF (10 mL) and cooled to -78°C. Then *n*-BuLi (3.1 mL, 1.6 M, 5 mmol) was added dropwise over a period of 5 min. After stirring for 40 min at -78°C diethyl phosphonomethyl triflate (1.6 g, 5 mmol) in THF (2 mL) was added. Stirring was continued for 30 min at -78°C and 2 h at RT. After this period TLC-analysis showed that the reaction had gone to completion. The reaction mixture was diluted with ether and washed wit 25% NH_4Cl, H_2O and dried over $MgSO_4$. Column chromatography over silica gel (elution: light petroleum/ethyl acetate 3/7 v/v) gave the title compound in 89% yield.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 14.7, 15.2, 15.4 16.4 (C13, C14, C15, OCH_2CH_3); 24.4 (C12); 25.0, 25.6 (C5, C9); 38.5, 38.6 (C4, C8); 60.8, 61.0 (OCH_2CH_3); 62.1 (d, OCH_2P, $J_{C,P}$ = 167.1 Hz); 67.9 (d, C1, $J_{C,P}$ = 11.7 Hz); 119.0 (C2); 122.6 (C6); 123.2 (C10); 129.7 (C11); 133.9 (C7); 140.2 (C3);

^1H NMR ($CDCl_3$) δ 1.32 (t, 6 H, OCH_2CH_3, J = 7.06 Hz); 1.57, 1.66 (2xs, 12 H, H12, H13, H14, H15); 1.84-2.11 (m(b), 8 H, H4, H5, H8, H9); 3.74 (d, 2 H, OCH_2P, $J_{H,P}$ = 8.48 Hz); 4.09 (d, 2 H, H1, $J_{1,2}$ = 6.63 Hz); 4.14 (dq, 4 H, OCH_2CH_3, J = 7.06 Hz, $J_{H,P}$ = 8.13 Hz); 5.00-5.14 (m(b), 2 H, H6, H10); 5.29 (t, 1 H, H2, $J_{2,1}$ = 6.66 Hz).

$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 21.9.

General procedure for the synthesis of phosphonomorpholidates (11)

The diester (7, 4 mmol) was refluxed overnight in the presence of 1N KOH (30 mL) and ethanol (30 mL). The ethanol was evaporated and after addition of CH_2Cl_2 (25 mL) the aqueous layer was acidified with 10% HCl. The mixture was stirred for 1 h and the organic layer was washed with brine, dried over $MgSO_4$ and concentrated to dryness. The oily residue was evaporated three times with toluene (10 mL) before it was redissolved in CH_2Cl_2 (10 mL) and brought under an argon atmosphere. Then *N,N*-diethyl-(trimethylsilyl)amine (1.9 mL, 7.6 mmol) was added and the reaction was allowed to stir for 1.5 h at RT. After evaporation of the solvent the residue was redissolved in toluene and evaporated. The remainder was dissolved in CH_2Cl_2 (10 mL) containing DMF (4 drops) and cooled to 0°C before oxalyl chloride (0.87 mL, 8 mmol) was added over a period of 5 min. Stirring was continued for 45 min at 0°C and 45 min at RT. Then the reaction mixture was concentrated and evaporated with toluene (3x10 mL). The residue was dissolved in THF (10 mL) and cooled to 0°C. Then morpholine (0.82 mL, 8 mmol) was added dropwise and after stirring for 2 h at 0°C the reaction was complete (TLC: 5% MeOH in CH_2Cl_2). The reaction mixture was diluted with ether and washed with H_2O, 10% $NaHCO_3$ and dried over $MgSO_4$. The organic layer was concentrated and the residue was applied to a column of silicagel. Elution with CH_2Cl_2/CH_3OH (100/0 → 95/5 v/v) gave the phosphonomorpholidates.

11a: yield: 86%. $^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.4, 15,9, 16.6, 17.1 (C13, C14, C15, OCH_2CH_3); 25.1 (C12); 25.9, 26.6 (C5, C9); 26.2 (d, C1, $J_{C,P}$ = 138.9 Hz); 39.2 (C4, C8); 43.8 (NCH_2 morpholine); 59.1 (OCH_2CH_3); 66.7 (OCH_2 morpholine); 112.4 (d, C2, $J_{C,P}$ = 8.8 Hz); 123.3 (C6); 123.8 (C10); 130.6 (C11); 134.6 (C7); 139.3 (d, C3, $J_{C,P}$ = 13.2 Hz).

^1H NMR ($CDCl_3$) δ 1.33 (t, 3 H; OCH_2CH_3); 1.60, 1.68 (2xs, 12 H, H12, H13, H14, H15); 1.92-2.17 (m, 8 H; H4, H5, H8, H9); 2.49 (dd, 2 H, H1, $J_{1,2}$= 7.3 Hz, $J_{H,P}$ = 22.4 Hz); 3.10-3.17 (m, 4 H, NCH_2 morpholine); 3.64-3.74 (m, 4 H, OCH_2 morpholine); 4.09 (q, 4 H, OCH_2CH_3); 5.04-5.23 (m, 3 H, H2, H6, H10).

$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 30.7

Anal. calcd. for $C_{21}H_{38}NO_3P$ (383.52): C 65.77, H 9.99, N 3.65, P 8.08; found C 65.56, H 10.03, N 3.60, P 8.05%

11b: yield: 92%. $^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.7, 17.4, 16,6 (C13, C14, C15, OCH_2CH_3); 25.4 (C12); 26.0, 26.5 (C5, C9); 39.5 (C4, C8); 44.1 (NCH_2 morpholine); 59.8 (d, OCH_2CH_3, $J_{C,P}$ = 7.3 Hz); 64.1 (d, OCH_2P, $J_{C,P}$ = 133.9 Hz); 67.0 (OCH_2 morpholine); 69.1 (d, C1, $J_{C,P}$ = 13.8 Hz); 119.6 (C2); 123.4 (C6); 124.0 (C10); 130.7 (C11); 135.1 (C7); 141.5 (C3).

^1H NMR ($CDCl_3$) δ 1.34 (t, 3 H, OCH_2CH_3, J = 7.19 Hz); 1.60, 1.68 (2xs, 12 H, H12, H13, H14, H15); 2.00-2.10 (m(b), 8 H, H4, H5, H8, H9); 3.17-3.25 (m, 4 H, NCH_2 morpholine); 3.64-3.78 (m, 6 H, OCH_2P and OCH_2 morpholine); 3.83-4.21 (m, 4 H, OCH_2CH_3, H1); 5.08-5.10 (m, 2 H, H6, H10); 5.30 (t, 1 H, H2, $J_{2,1}$ = 6.93 Hz).

$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 24.8.

Anal. calcd. for $C_{22}H_{40}NO_4P$ (413.54): C 63.90, H 9.75, N 3.39, P 7.49; found C 63.62, H 9.64, N 3.42, P 7.21%

General procedure for the synthesis of phosphonophosphates and phosphono-methylphosphonates (3, 4, 14 and 15)

The morpholidate (0.7 mmol) was evaporated three times with toluene (5 mL) and dissolved in acetonitrile (5 mL). Then trimethylsilyl bromide (145 μL, 1.1 mmol) was added and the reaction was stirred at RT. When ^{31}P NMR showed that the reaction had gone to completion, the reaction mixture was concentrated and the residue was evaporated three times with toluene (5 mL). The residue was redissolved in THF (5 mL) and tetra-*n*-butylammonium fluoride (1M in THF, 0.8 mL) was added at RT. The reaction was monitored again by ^{31}P NMR and after the appropriate time, the reaction mixture was concentrated and the residue was evaporated three times with pyridine (5 mL). The crude deprotected morpholidate was dissolved in pyridine (1 mL) and a solution of phosphoric acid (mono tributylammonium salt, 2.1 mmol) or methylphosphonic acid (mono tributylammonium salt, 2.1 mmol) in pyridine (1 mL) was added. After stirring for 48 h at room temperature the solvent was removed *in vacuo* and the residue was dissolved in water and applied to a Dowex 50Wx4 column (NH_4^+ form). The eluate was lyophilized and the residue was purified on a CHP20P column, that was eluted with a linear gradient of acetonitrile in water. Concentration of the appropriate fractions afforded the title compounds as their ammonium salts.

3: yield: 51%. $^{13}C\{^1H\}$ NMR (D_2O) δ 16.4 (C14); 16.8 (C13); 18.0 (C15); 26.0 (C12); 27.1 (C5); 27.3 (C9); 40.3 C4, C8); 66.6 (d, OCH_2P, $J_{C,P}$ = 164 Hz); 69.8 (d, C1, $J_{C,P}$ = 11.8 Hz); 120.4 (C2); 124.4 (C6); 125.2 (C10); 131.7 (C11); 135.8 (C7); 143.3 (C3);

^1H NMR (D_2O) δ 1.56 (s, 3 H, H15); 1.58 (s, 3 H, H14); 1.63 (s, 3 H, H12); 1.70 (s, 3 H, H13); 1.91-2.09 (m, 8 H, H4, H5, H8, H9); 3.70 (d, 2 H, OCH_2P, $J_{H,P}$ = 8.87 Hz); 4.14 (d, 2 H, H1, $J_{1,2}$ = 6.73 Hz); 5.06-5.12 (m, 2 H, H6, H10); 5.38 (t(b), 1 H, H2, $J_{2,1}$ = 6.62 Hz).

$^{31}P\{^1H\}$ NMR (D_2O) δ 16.26 ($J_{P,P}$ = 29.0 Hz); 17.59 ($J_{P,P}$ = 29.0 Hz);

Anal. calcd. for $C_{16}H_{39}N_3O_7P_2$ (447.45): C 42.95, H 8.79, N 9.39, P 13.85; found C 43.01, H 8.90, N 9.35, P 13.66%

4: yield: 49%. $^{13}C\{^1H\}$ NMR (D_2O) δ 14.5 (d, CH_3P, $J_{C,P}$ = 139.2 Hz); 16.2, 16.6, 17.9 (C13, C14, C15); 25.9 (C12); 26.9 (C5); 27.1 (C9); 40.1 (C4, C8); 66.4 (d, CH_2P, $J_{C,P}$ = 164.0 Hz); 69.5 (d, C1, $J_{C,P}$ = 11.7 Hz); 120.1 (C2); 124.6 (C6); 125.0 (C10); 131.7 (C11); 135.8 (C7); 143.2 (C3).

^1H NMR (D_2O) δ 1.46 (d, 3 H, CH_3P, $J_{H,P}$ = 17.20 Hz); 1.60 (s, 3 H, H15); 1.62 (s, 3 H, H14); 1.67 (s, 3 H, H12); 1.73 (s, 3 H, H13); 1.96-2.59 (m, 8 H, H4, H5, H8, H9); 3.70 (d, 2 H, OCH_2P, $J_{H,P}$ = 8.83 Hz); 4.17 (d, 2 H, H1, $J_{1,2}$ = 7.08 Hz); 5.11 (t, b, 1 H, H10, $J_{10,9}$ = 6.59 Hz); 5.15 (t, b, 1 H, H6, $J_{6,5}$ = 6.26 Hz); 5.41 (t, b, 1 H, H2, $J_{2,1}$ = 7.03 Hz).

$^{31}P\{^1H\}$ NMR (D_2O) δ 9.25 (d, OCH_2P, $J_{P,P}$ = 29.6 Hz); 18.37 (d, CH_3P, $J_{P,P}$ = 29.6 Hz).
Anal. calcd. for $C_{17}H_{38}N_2O_6P_2$ (428.45): C 47.66, H 8.94, N 6.54, P 14.46; found C 47.54, H 8.81, N 6.32, P 14.31%

14: yield: 70%. $^{13}C\{^1H\}$ NMR (D_2O) δ 16.0 (C14); 16.3 (C13); 17.6 (C15); 25.4 (C12); 26.9 (d, C1, $J_{C,P}$ = 137.0 Hz); 27.0 (C5 and C9); 40.0 (C4 and C8); 116.2 (d, C2, $J_{C,P}$ = 10.3 Hz); 125.1 (C6); 125.2 (C10); 131.8 (C11); 135.4 (C7); 139.3 (d, C3, $J_{C,P}$ = 15.5 Hz);
^1H NMR (D_2O) δ 1.59, 1.66 (2xs, 12 H, H12, H13, H14, H15); 1.89-2.76 (m, 8 H, H4, H5, H8, H9); 2.58 (m, 2 H, H1); 5.11-5.24 (m, 3 H, H2, H6, H10);
$^{31}P\{^1H\}$ NMR (D_2O) δ -8.81 ($J_{P,P}$ = 24.4 Hz); 16.92 ($J_{P,P}$ = 24.5 Hz)

15: yield: 69%. $^{13}C\{^1H\}$ NMR (CD_3OD) δ 14.3 (d, CH_3P, $J_{C,P}$ = 138.5 Hz); 16.1 (C14); 16.3 (C13); 17.8 (C15); 25.8 (C12); 27.1 (d, C1, $J_{C,P}$ = 136.6 Hz); 27.1 (C5 and C9); 40.1 (C4 and C8); 115.7 (d, C2, $J_{C,P}$ = 10.2 Hz); 125.0 (C6 and C10); 131.7 (C11); 135.6 (C7); 139.2 (d, C3, $J_{C,P}$ = 14.7 Hz);
^1H NMR (CD_3OD) δ 1.42 (3H, d, $J_{P,H}$ = 17.22 Hz, CH_3P); 1.60 (s, 6 H, H14, H15); 1.67 (s, 6 H, H12, H13); 1.85-2.18 (m, 8 H, H4, H5, H8, H9); 2.43-2.61 (m, 2 H, H1); 5.01-5.22, 5.31-5.43 (m, 3 H, H2, H6, H10).
$^{31}P\{^1H\}$ NMR (CD_3OD) δ 17.0 (dd, $J_{P,P}$ = 29.3 Hz)

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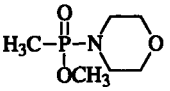
14 Methyl methylphosphonomorpholidate = 

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ADDENDUM

Different Analogues of Farnesyl Pyrophosphate Inhibit Squalene Synthase and Protein:farnesyltransferase to Different Extents[1]

Abstract: Farnesyl pyrophosphate analogues were investigated for their possible differential influence on cholesterol synthesis and on protein prenylation by testing their inhibitory potency on two farnesyl pyrophosphate-consuming enzymes, squalene synthase, a secondary regulation site in the cholesterol synthesis pathway, and protein:farnesyl transferase, which e.g. plays an important role in the function of Ras-proteins. For the transferase determination a rapid in vitro assay, using Sepharose-bound Ras-peptides, has been developed. The distinct farnesyl pyrophosphate analogues showed a different order of potency in the inhibition of these two enzymes. Using the farnesyl transferase assay with pre-$p21^{Ha\text{-}ras}$ as substrate the same result was obtained. The difference observed in the in vitro assay was also reflected in the inhibition of cholesterol synthesis and of protein prenylation in Rat-1.H-ras13 cells, a rat fibroblast cell line that overproduces human $p21^{Ha\text{-}ras}$. The inhibition of the farnesylation of Ha-ras by the most potent compound was shown to be concentration-dependent in these cells. This work shows that farnesyl pyrophosphate analogues can be developed for specific inhibition of different processes such as cholesterol synthesis and protein prenylation.

Introduction

Inhibitors of the cholesterol biosynthesis are currently in use as cholesterol lowering drugs. The vastatins, potent and specific inhibitors of the rate limiting enzyme of this pathway, i.e. HMG-CoA reductase (EC 1.1.1.34), are well known examples.[2] However, it has been shown that these compounds can influence other processes which are dependent on the synthesis of isoprene intermediates of the cholesterol biosynthesis pathway as well. Of these, the posttranslational modification of members of certain protein families by covalent linkage of a farnesyl- or geranylgeranyl-group to a cysteine residue by the enzymes protein:farnesyl transferase[3] and protein:geranylgeranyl transferase,[4] respectively, shortly addressed as protein (iso)prenylation, has attracted much attention (for reviews: refs. 5-7). For instance, the farnesylation of $p21^{ras}$ was shown to play a role in the interaction with its site of action in the plasma membrane[8] and possibly in protein-protein recognition.[9] Furthermore, prevention of farnesylation by inhibiting HMG-CoA reductase abolished the transforming potency of mutated $p21^{ras}$.[10] It is preferable to develop inhibitors of cholesterol synthesis as cholesterol lowering drugs, which do not interfere with protein isoprenylation, as well as to develop specific inhibitors of the latter process[11,12] without

influencing cholesterol biosynthesis. Squalene synthase (EC 2.5.1.21) is thought to be a good target for such cholesterol synthesis inhibitors. Besides the very recent discovery of the squalestatins/zaragozic acids,[13,14] analogues of farnesyl pyrophosphate (FPP), the substrate of this enzyme, have been developed as inhibitors of squalene synthase.[15,16] However, FPP is also the substrate of protein:farnesyltransferase and indirectly, via the enzyme geranylgeranyl pyrophosphate synthase, of protein-geranylgeranylation.

Indeed, a few FPP analogues have been shown to inhibit protein-farnesylation.[17,18] In this report we have looked for differences in inhibitory potency of several FPP analogues in both pathways by studying their influence on squalene synthase and protein:farnesyltransferase activity in vitro and show that analogues of FPP can be developed that are different in their inhibitory action towards these processes.

Materials and Methods

Synthesis of farnesyl pyrophosphate analogues

Six different farnesyl pyrophosphate analogues (FPPA 1-6; see Table 1 for chemical structures) were synthesized according to the procedures described by Kolodyazhnyi *et al.* for FPPA 1,[19] Valentijn *et al.* for FPPA 2-4[20] and by Biller *et al.* for FPPA 5.[16] FPPA 6 was synthesized according to a modification of the procedure of Valentijn *et al.* (to be published elsewhere).

Squalene synthase assay in rat liver microsomal preparations

Squalene synthase activity was determined in rat liver microsomal preparations according to a modification of a previously described procedure.[21] which shortened the assay time considerably. Incubation conditions remained unchanged, however the extraction and TLC separation of squalene was replaced by the following procedure: after the reaction was stopped by addition of 150 µl 5 M NaOH, [^{3}H]-Squalene (80,000 dpm) as a recovery standard, unlabelled squalene (5 µg) as carrier and subsequently 1 ml of chloroform/methanol (1:2) were added. Vigorously mixing resulted in a homogeneous organic/anorganic phase containing the lipids in a soluble form. Following centrifugation to remove precipitated proteins the supernatant was loaded onto a prepacked Amprep octadecyl C18 column (Amersham). After washing the column with 4 ml of methanol/20 mM NaOH (1:1), ^{3}H/^{14}C-labelled squalene was eluted with 2 ml of hexane into a scintillation vial and the radioactivity determined in a Tricarb liquid scintillation counter (Packard). The ^{14}C-cpm of the squalene formed was corrected for the recovery of [^{3}H]-squalene, which was between 70 and 90%.

Assay of protein:farnesyltransferase activity using a C-terminal peptide of pre-p21$^{N\text{-}Ras}$ coupled to Sepharose beads as substrate

Two peptides, peptide A containing the carboxyl-terminal amino acid sequence of human pre-p21$^{N\text{-}Ras}$, i.e.

NH_2-Aca-Met-Gly-Leu-Pro-Cys-Val-Val-Met-COOH

|

S-*tert*-butyl

(Aca = ε-aminocaproic acid), and peptide B (control peptide; peptide A with Cys replaced by Ala) with sequence NH_2-Aca-Met-Gly-Leu-Pro-Ala-Val-Val-Met-COOH were coupled to CH-Sepharose 4B (Pharmacia) through the sole amino-group according to the manufacturer's instructions. The thiol of the cysteine-residue of peptide A was protected by a *tert*-butylthio-group, which was removed before use in the assay by reduction with DTT. The coupled peptides are designated pepAsep and pepBsep, respectively. Using pepAsep as substrate and pepBsep as control the assay of protein:farnesyltransferase activity was performed in the presence of various concentrations of FPPAs as indicated in Fig. 2A. Twenty-five µl of a mixture containing 5 µl of pepAsep or pepBsep (containing 80 pmol of peptides), 13 µl rabbit reticulocyte lysate (Promega) used as enzyme source,[22] 0.5 mM $MgCl_2$, 1 mM DTT, 50 mM Tris-HCl (pH 7.4) and 0.7 µM [^{3}H]-FPP (spec. radioact. 15 Ci/mmol; American Radiolabeled Chemicals, USA) were incubated at 37°C for 30 min under continuous shaking. The reaction was terminated by addition of 1 ml of 2% SDS and the beads were washed 3 times with 2% SDS under shaking for 45 min at 50°C. The radioactivity bound to the sepharose, as determined in a Packard Tricarb liquid scintillation counter, was strongly dependent on the presence of the cysteine residue in peptide A (compare e.g. values in legend of Fig. 2A). For the calculation of the protein:farnesyltransferase activity the ^{3}H-counts bound to pepBsep are subtracted from the counts bound to pepAsep.

Assay of protein:farnesyltransferase activity using pre-p21$^{Ha\text{-}Ras}$ as substrate

Protein:farnesyltransferase assay was performed essentially according to Reiss *et al.*[23] with a slight modification: the 25 µl-incubation mixture contained 0.5 µg recombinant pre-p21$^{Ha\text{-}Ras}$, 0.5 mM $MgCl_2$, 1 mM DTT, 50 mM Tris-HCl (pH 7.4), 0.7 µM [^{3}H]-FPP (American Radiolabeled Chemicals), 13 µl rabbit reticulocyte lysate (Promega) and various concentrations of FPPAs as indicated in Fig. 2B and was incubated for 30 min at 37°C.

Culture of and determination of protein isoprenylation in Rat-1.H-ras13 cells

Rat-1.H-ras13 cells[24] were cultured in 10 cm^2 wells in DMEM, supplemented with 10% foetal calf serum. For determination of protein prenylation and the influence of FPPAs on that process the cells were pre-incubated in DMEM containing 1% human serum albumin, 2.5 µl of simvastatin, and 0-200 µM of FPPA 1 or FPPA 5 for 2 h at 37°C in a 5% CO_2 atmosphere. Incubation for 18 h was started by addition of 20 µl (20 µCi) of [^{3}H]-mevalonolactone (American Radiolabeled Chemicals, USA, spec. radioact. 50 Ci/mmol). After incubation the cells were lysed in 300 µl PBS supplemented with 1% Triton X-100, 0.5% sodium deoxycholate, and the protease inhibitors phenylmethylsulfonylfluoride (1 mM), leupeptine (50 µg/ml), pepstatin A (50 µg/ml) and trasylol (300 K.I.U./ml), collected and stored at -20°C. For analysis of the ^{3}H-labelled polypeptides, the lysates were thawed, centrifuged (13,000 rpm for 15 min at 4°C) and the polypeptides precipitated from the supernatant by adding acetone/NH_4OH (18:1). The precipitate was dissolved in 75 µl of SDS-sample buffer (2.5% SDS, 5% β-mercaptoethanol, 10 mM Tris (pH 8.0), 1 mM EDTA, 10% glycerol, 0.005% bromophenol blue) and electrophoresis was performed on a 14% polyacrylamide slab gel according to Laemmli *et al.*.[25] Labelled bands were visualized by fluorography.

Immunodetection of $p21^{Ras}$-farnesylation in Rat-1.H-ras13 cells

For assaying the influence of FPPAs on the farnesylation of $p21^{Ha\text{-}ras}$ in H-ras13 cells, $p21^{Ha\text{-}ras}$ was collected from the cell lysates by using the monoclonal antibody Y13-259 coupled to protein-G-Sepharose (Pharmacia) as described by Osterop *et al.*.[26] The immuneprecipitated polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography.

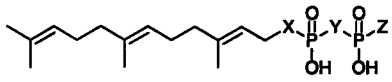
Determination of cholesterol synthesis in Rat-1.H-ras13 cells

One day prior to the measurements the culture medium was replaced by 1 ml of DMEM, supplemented with 10% of human lipoprotein deficient serum. Determination of the cholesterol synthesis in H-ras13 cells in the absence or presence of 50 µM or 200 µM of FPPA 1 or FPPA 5 was performed according to a modification of a previously described method.[27] After the cells had been incubated for 2 h with the same medium additionally containing the FPPAs [^{14}C]-acetate (Amersham; spec. rad. act. 55 mCi/mmol) was added (0.4 µCi/well containing 1 ml of medium). The incubation was continued for 18 h and then the medium was removed, the cells were lysed in 300 µl of 0.2 M NaOH and subsequently neutralized with 30 µl 2 M HCl. Media and cell lysates were stored at -20°C. After thawing samples were taken for protein determination, lipids extracted from cell lysate and medium together, saponified, and cholesterol purified in t.l.c. system I as described previously.[27] The ^{14}C-radioactivity incorporated into cholesterol was counted in a Tri-carb liquid scintillation analyzer (Packard), corrected for the recovery of [^{3}H]-cholesterol and expressed as ^{14}C-dpm/mg of cellular protein. Values are the average of duplicate cell incubations. The data presented are expressed as percentages of the control values, as means ± S.E.M., obtained from 3 separately performed experiments.

Results and discussion

Inhibition of squalene synthase activity by farnesyl pyrophosphate analogues

Five FPP analogues shown in Table 1 (FPPA 1-5) were tested for inhibition of squalene



Compound	X	Y	Z
FPP	-O-	-O-	-OH
FPPA 1	-	-O-	-OH
FPPA 2	-	-O-	$-CH_3$
FPPA 3	$-CH_2-$	-O-	-OH
FPPA 4	$-CH_2-$	-O-	$-CH_3$
FPPA 5	$-OCH_2-$	$-CH_2-$	-OH
FPPA 6	$-OCH_2-$	-O-	-OH

Table 1. Chemical structure of the farnesyl pyrophosphate analogues.

synthase activity in rat liver microsomal preparations. The results as depicted in Fig. 1 are in general terms in agreement with earlier reports by Biller *et al.*[15,16] and show that an

	IC_{50}-values (μM) (mean ± S.E.M.; n = 3)	
Compound	Squalene synthase	N-ras-peptide farnesylation
FPPA 1	129 ± 11	0.34 ± 0.05
FPPA 2	n.i.*	n.d.**
FPPA 3	518 ± 12	0.93 ± 0.23
FPPA 4	> 500	n.d.
FPPA 5	0.68 ± 0.14	7.15 ± 2.32

*n.i. = non-inhibitory.

**n.d. = not determined.

Table 2. IC_{50}-values for inhibition of squalene synthase and N-ras-peptide:farnesyl-transferase by farnesyl pyrophosphate analogues.

IC_{50}-values were determined from the inhibitor curves obtained from separately performed experiments by means of curve fitting and mathematical calculation of the 50% inhibition value. Means ± S.E.M. from three experiments are given. The composite figures of the squalene synthase and protein:farnesyltransferase inhibitor curves, used for this calculation, are depicted in Figs. 1 and 2A, respectively.

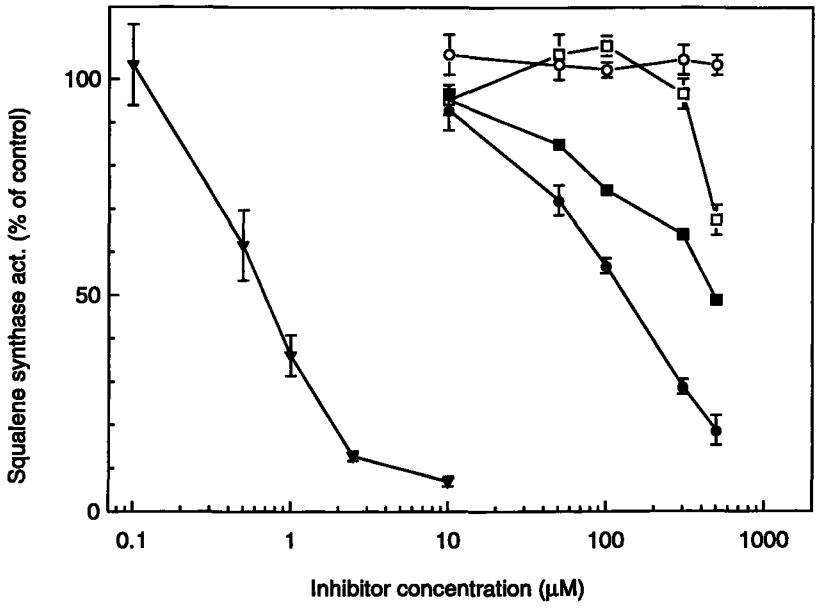


Figure 1. Inhibition of squalene synthase by farnesyl pyrophosphate analogues
Squalene synthase activity was determined in rat liver microsomal preparations in the presence of the indicated concentrations of FPPA 1 (•), FPPA 2 (○), FPPA 3 (■), FPPA 4 (□) and FPPA 5 (▾). The enzyme assay was performed in duplicate determinations in three separate experiments (bars indicate S.E.M.) and the activity is expressed as percentage of control values, which were in the range of 1.7-2.9 nmol/min/mg of microsomal protein.

ether-oxygen at X and methylene group at Y in FPPA 5 strongly potentiated the inhibitory capacity compared to the phosphonate analogue FPPA 3 (an increase of 3 orders of magnitude). FPPA 1, which is one carbon-atom shorter than FPPA 3, is only about five times more potent. Replacement of the charged oxygen on the terminal phosphate with a methyl group (FPPA 2 and 4) strongly diminished the inhibitory potential. Therefore, as reflected by the IC_{50}-values (Table 2), the order of potency is: FPPA 5 ≫ FPPA 1 > FPPA 3 > FPPA 4 ≫ FPPA 2.

Inhibition of protein:farnesyltransferase activity by farnesyl pyrophosphate analogues
The most potent squalene synthase inhibitors, compounds 1, 3 and 5, were tested for their ability to inhibit the protein:farnesyltransferase activity in rabbit reticulocyte lysates. The assay was performed using a sepharose-coupled peptide substrate as described under Materials and Methods in the presence of various concentrations of the FPPAs. As is

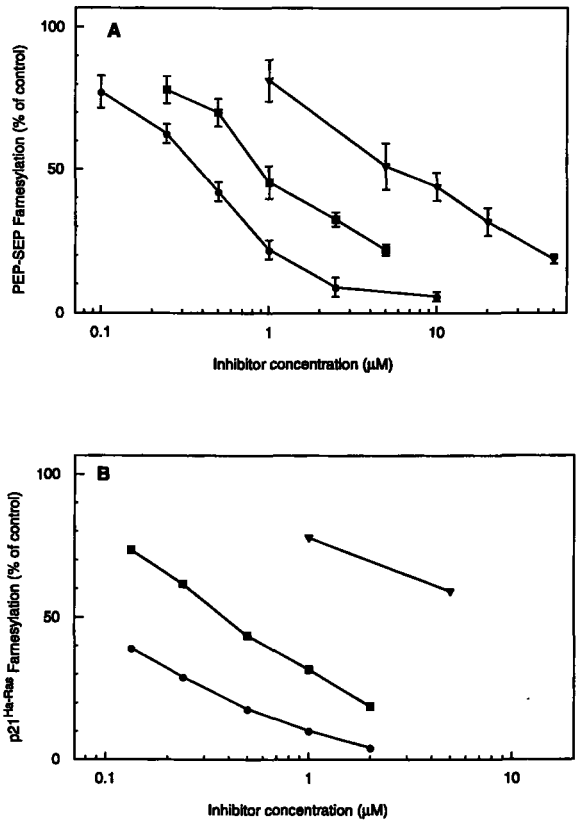


Figure 2. Inhibition of protein:farnesyltransferase by farnesyl pyrophosphate analogues, using (A) sepharose-bound N-ras peptides or (B) pre-p21$^{Ha\text{-}ras}$ as substrate
A) Peptide substrates, pepAsep and pepBsep, or B) pre-p21$^{Ha\text{-}ras}$ were incubated with reticulocyte lysate in the presence of 0.7 μM of [^{3}H]-FPP and the indicated concentrations of the farnesyl pyrophosphate analogues FPPA 1 (•), 3 (■) and 5 (▾). As described under Materials and Methods the peptide-farnesylation activity (A) was calculated as ^{3}H-dpm bound to pepAsep minus the pepBsep-bound dpm and expressed as percentage of control (control values pepAsep 12740 ± 2060 dpm, pepBsep 670 ± 290 dpm). The data shown are mean values ± S.E.M. from three separately performed experiments. Using pre-p21$^{Ha\text{-}ras}$ as substrate (B) the [^{3}H]-radioactivity bound to the protein precipitate is expressed as percentage of control (16110 ± 880 dpm).

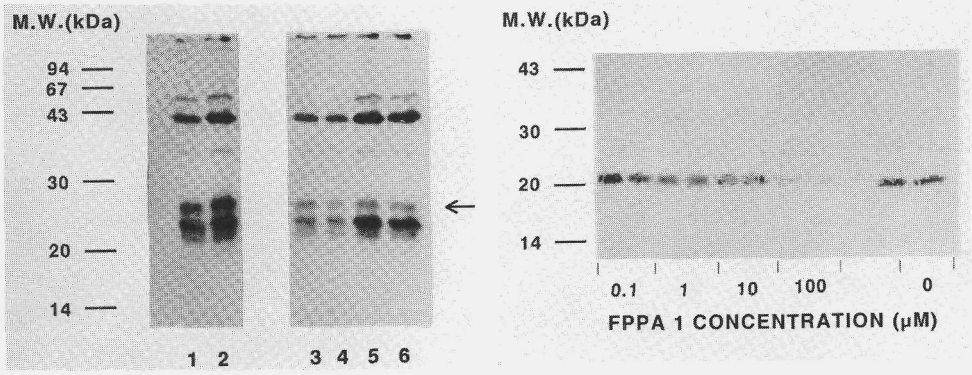
depicted in Fig. 2A all three compounds are inhibitors of the farnesylation reaction. However the order of potency, FPPA 1 > FPPA 3 > FPPA 5 (IC_{50}-values are given in Table 2), was different from that in the squalene synthase reaction. The relative diminished potency of FPPA 5 in this case may suggest an important role for the oxygen atom in position Y, which is present in FPPA 1 and 3 but not in 5, in the inhibition of the enzyme. Therefore we tested another FPPA, FPPA 6, which was the same as FPPA 5, except that the CH_2-group, denoted as Y in Table 1, was replaced by an oxygen atom, in the peptide-

farnesylation reaction. Indeed FPPA 6 was a stronger inhibitor (IC_{50} = 0.5 μM) than FPPA 5 and almost as strong as FPPA 1 (data not shown).

In order to verify the results obtained using the sepharose-coupled peptides, the farnesylation reaction was also performed using a biological substrate, in this case pre-$p21^{Ha\text{-}Ras}$, and the inhibitors FPPA 1, 3 and 5. As shown in Fig. 2B the order of inhibitory potency in this reaction was the same as that in the assay with the bead-bound peptide. This demonstrates that the latter assay will be useful for rapid screening of inhibitors of protein:farnesyltransferase.

Inhibition of protein-isoprenylation by FPPAs in Rat-1.H-ras13 cells in culture

In order to show that these compounds can also act in cultured cells, Rat-1.H-ras13 cells, which overexpress human $p21^{Ha\text{-}ras}$,[24] were incubated with 200 μM of either FPPA 1 or 5 in the presence of [^{3}H]-mevalonate as source for the label in protein-isoprenyl groups and 2.5 μM of simvastatin to inhibit endogenous mevalonate production. After incubation, the labelled isoprenylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. The result is shown in Fig. 3A. Compared to control incubations (Fig. 3A, lanes 1,2) FPPA 1 prevented the incorporation of [^{3}H]-mevalonate into a number of proteins (Fig. 3A, lanes 3,4), showing its inhibitory action in cultured cells as well. Since the majority of mammalian prenylated proteins bear the geranylgeranyl-group,[28,29] it seems likely that FPPA 1 also inhibits the geranylgeranylation process, possibly via the inhibition of geranylgeranyl pyrophosphate synthase, which uses FPP as substrate as well. On the other hand, FPPA 5 hardly inhibited protein isoprenylation, except that a 26-kD band (arrow in Fig. 3A) seems to be reduced as compared to the control pattern. These results are in concordance with the differences in inhibitory potency of the compounds in the in vitro protein-farnesylation. However, it cannot be precluded that these results are, in part, due to a differences in their uptake by the H-ras13 cells. Protein synthesis in these cells (measured as the incorporation of [^{35}S]-methionine into protein) was not influenced by these FPPAs (results not shown). Further, the effect of FPPA 1 on the farnesylation of $p21^{Ha\text{-}ras}$ in these cells was also determined. H-ras13 cells were incubated with [^{3}H]-mevalonate and 0.1-100 μM of FPPA 1 for 24 h and $p21^{ras}$ was collected on immobilized monoclonal antibody Y13-259. After separation by SDS polyacrylamide gel electrophoresis the [^{3}H]-labelled polypeptides were visualized by autoradiography. In Fig. 3B it is shown that with increasing FPPA 1 concentrations less radioactivity was incorporated into $p21^{ras}$. This decrease in farnesylation started only at FPPA 1 concentrations higher than 1 μM and may indicate that this compound is not easily taken up by H-ras13 cells.



A B

Figure 3. Inhibition of protein prenylation in Rat-1.H-ras13 cells by farnesyl pyrophosphate analogues

A) H-ras13 cells were incubated for 20 h in the absence (lanes 1 and 2) or presence of 200 μM of FPPA 1 (lanes 3 and 4) or FPPA 5 (lanes 5 and 6). After the first 2 h, 20 μCi of [^{3}H]-mevalonate was added (see Materials and Methods for incubation conditions). [^{3}H]-labelled proteins were precipitated with acetone/ammonia, analyzed by SDS-polyacrylamide (14%) gel electrophoresis and visualized by fluorography. Molecular weight markers indicate the size of the polypeptides shown.

B) H-ras13 cells were incubated with the indicated concentrations of FPPA 1 for 20 h and with 20 μCi of [^{3}H]-mevalonate for the last 18 h. Cells were lysed and [^{3}H]-p21$^{Ha\text{-}ras}$ was collected on sepharose-coupled antibody Y13-259. The precipitate was analyzed by electrophoresis on a 14% polyacrylamide slab gel and the radiolabelled bands were visualized by fluorography.

The effect of FPPA 1 and FPPA 5 on the cholesterol synthesis in H.ras13 cells was examined as well. As shown in Table 3, FPPA 1 has no significant inhibitory effect on the [^{14}C]-acetate incorporation into cholesterol. On the other hand FPPA 5 inhibited cholesterol synthesis to about 45% at the highest concentration used. This is in agreement with the in vitro potencies of both compounds in inhibiting squalene synthase activity (Fig. 1, Table 2).

Our results show that FPPAs can have different relative potencies in their inhibitory action towards different FPP-consuming enzymes as we herewith have shown for squalene synthase and protein:farnesyltransferase. While the compounds described here are all negatively charged and therefore may have limited cellular uptake, modifications, such as the addition of masking groups, which are removed within the cell, can be considered.

FPPA concentration	Cholesterol synthesis (% of control values) (mean ± S.E.M.; n = 3)	
	FPPA 1	FPPA 5
-	100	100
50 μM	92.4 ± 4.5	71.7 ± 17.4
200 μM	93.0 ± 2.9	55.5 ± 10.2

Table 3. Inhibition of cholesterol synthesis by farnesyl pyrophosphate analogues in Rat-1.H-ras13 cells.
H-ras13 cells were preincubated for 24 h in DMEM/10% lipoprotein deficient serum and incubated with 50 or 200 μM of FPPA 1 or FPPA 5 for 20 h. Two hours after the addition of the analogues 0.4 μCi of [^{14}C]-acetate/ml of culture medium was added and at the end of the incubation the incorporation of ^{14}C-counts into cholesterol was determined as described under Materials and Methods. The values (mean ± S.E.M.; n = 3) are expressed as percentage of controls (238,840 ± 72,190 dpm/mg of cellular protein).

Recently it has been published that a very potent inhibitor of squalene synthase, zaragozic acid[14] is, to a lesser extent, also an inhibitor of protein:farnesyltransferase in vitro, but not in cell culture.[30] Further, α-hydroxyfarnesylphosphonic acid, another FPPA has been reported to be a strong inhibitor of the farnesyltransferase activity[18] but also of squalene synthase.[30] The potency of yet another FPPA, [(farnesylmethyl)hydroxyphosphinyl] methyl] phosphonic acid, to inhibit protein:farnesyltransferase was much lower than that of α-hydroxyfarnesylphosphonic acid.[18] These and our observations show that FPPAs can be developed as specific and potent inhibitors for different processes, such as cholesterol synthesis and protein-farnesylation.

As shown in Fig. 3A, FPPA 1 and maybe more specifically also FPPA 5 may influence protein-geranylgeranylation. Several enzymes are involved in this process, such as geranylgeranyl pyrophosphate synthase,[31] protein:geranylgeranyltransferase-I[32,33] and protein:geranylgeranyltransferase-II.[32,34] It is therefore possible that these and other FPPAs or analogues of geranylgeranyl pyrophosphate will possess different specificities in the inhibition of these enzymes. This is currently under investigation.

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CHAPTER 3

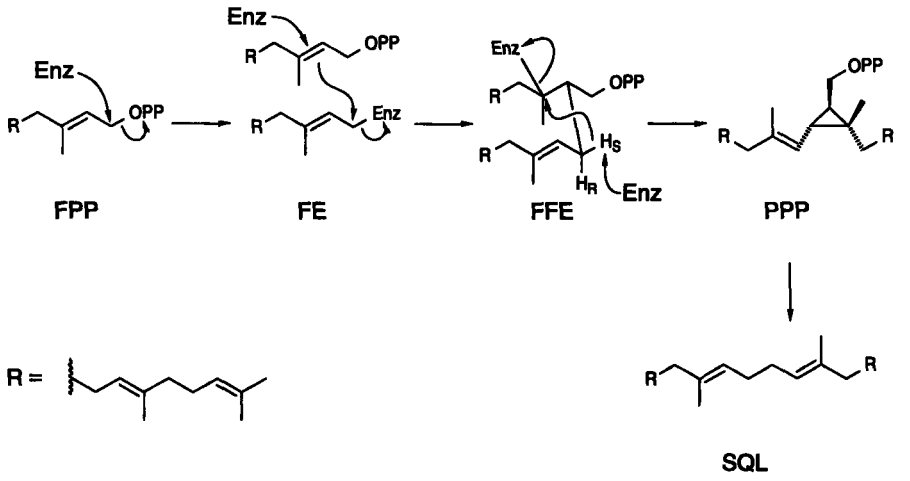
Synthesis of a Potential Enzyme-Specific Inhibitor of Squalene Synthase[1]

Abstract: A multi-step synthesis of an analogue (**12**) of a proposed intermediate (*i.e.* FFE) in the enzymatic conversion of farnesyl pyrophosphate to squalene is described. In addition evidence is presented for the inhibitory effect of **12** on squalene synthase.

Introduction

Squalene synthase (SS) is the first pathway-specific enzyme in the biosynthesis of cholesterol and catalyses, as depicted in Scheme 1, the head-to-head condensation of two molecules of farnesyl pyrophosphate (FPP) to squalene (SQL). The precise mechanism of the enzymatic conversion is not fully understood, but it has been implied that a ping-pong reaction[2] is involved in the transformation of FPP into PPP. The first step comprises replacement of the pyrophosphate moiety of one FPP by a nucleophilic group of SS giving a farnesyl-enzyme intermediate (FE). The C2-C3 double bond of a second FPP is then activated by another nucleophilic group of SS, followed by attack on the C1' of FE. The

Scheme 1



resulting compound (FFE) is cyclisized to presqualene pyrophosphate (PPP) by stereospecific abstraction of the pro-S proton at C1', followed by nucleophilic attack on C3. Up to now, different classes of compounds have been proposed to inhibit the biosynthesis of cholesterol. For instance, a major class of inhibitors comprises FPP-analogues,[3a-e] However, this type of compounds lack specificity, and are also potential inhibitors of other FPP consuming enzymes (*e.g.* geranylgeranyl pyrophosphate synthase or protein:farnesyl transferase). In order to increase enzyme specificity, the synthesis of analogues of PPP was reported[4]. Moreover, cationic mimics of FFE, based on the mechanism in Scheme 1, were recently described by Steiger[5] and Oehlschlager.[6]

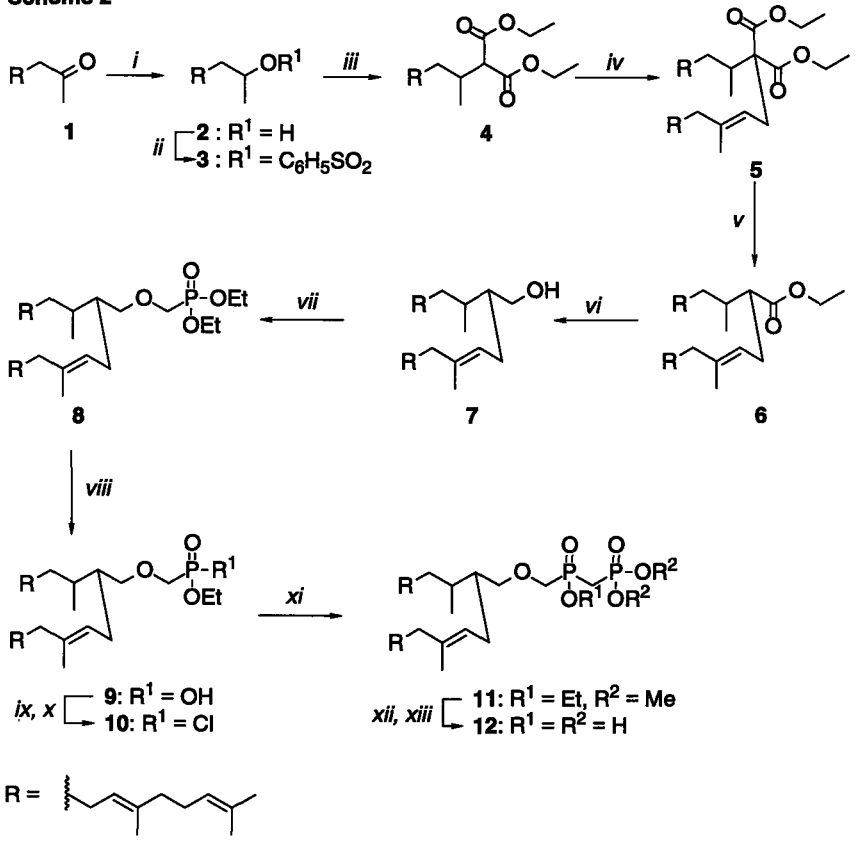
As part of a program[3d] directed towards the design and synthesis of inhibitors of the cholesterol biosynthesis, we here describe the preparation of a presumed enzyme-specific analogue of FFE (*i.e.* compound **12**).

Results and discussion

The synthetic route to the target compound **12** comprises, as outlined in Scheme 2, the following two distinct stages. The first stage deals with the build-up of the carbon skeleton and commences with reduction of geranylacetone[7] (**1**) in ether to give, after purification by silica gel chromatography, racemic (E)-6,10-dimethyl-5,9-undecadiene-2-ol (**2**) in excellent yield. An attempt to separate the individual isomers of alcohol **2** by derivatization with the chiral auxiliaries menthoxy acetyl chloride or camphorsulfonyl chloride was abortive. Sulfonylation of **2** with benzenesulfonyl chloride in pyridine produced **3**, which was used for the alkylation of the sodium enolate of diethyl malonate in tetrahydrofuran at 68°C, to give **4**. In a similar way, alkylation of **4** in tetrahydrofuran with farnesyl bromide in the presence of sodium hydride afforded homogenous **5**. Decarboxylation[8] of **5** was effected by the action of excess sodium cyanide in refluxing dimethyl sulfoxide, to yield the mono-ester derivative **6** as a mixture of diastereoisomers. ^{1}H and ^{13}C NMR spectroscopic analysis of **6** revealed that the decarboxylation process had proceeded without isomerisation of the double bonds.[8,9] Reduction of **6** resulted in the formation of target alcohol **7** which was isolated as an oil in 26% overall yield over the six steps.

The phosphinylphosphonate functionality was introduced by the following sequence of reactions. The alkoxide of **7**, generated *in situ* by the action of *n*-butyllithium in tetrahydrofuran at -78°C, was treated with diethyl phosphonomethyl triflate,[10] to give the protected phosphonate **8** in satisfactory yield (δ_P 21.8 ppm). Hydrolysis of diester **8** to its corresponding monoester, by refluxing for 16 h in a mixture of ethanol and 1N potassium hydroxide, furnished **9**, as gauged by ^{31}P NMR spectroscopy (δ_P 17.4 ppm). Introduction

Scheme 2[a]



[a]Reagents and conditions

i: $LiAlH_4$, ether, reflux (97%); *ii*: benzenesulfonyl chloride (74%); *iii*: diethylmalonate, NaH (79%); *iv*: NaH, farnesyl bromide (82%); *v*: NaCN, DMSO, reflux (73%); *vi*: $LiAlH_4$, ether (76%); *vii*: *n*-BuLi, $TfOCH_2P(O)(OEt)_2$ (84%); *viii*: KOH, ethanol, H_2O, reflux (96%); *ix*: $TMSNEt_2$, CH_2Cl_2; *x*: oxalyl chloride, DMF, CH_2Cl_2, 0°C; *xi*: $LiCH_2P(O)(OMe)_2$, -78°C (59%, 3 steps); *xii*: TMSBr, 2,4,6-collidine, CH_2Cl_2, 0°C; *xiii*: NH_3, H_2O, dioxane (57%).

of the second phosphonate was executed by the following three-step procedure.[11] Thus, silylation of crude **9** with *N,N*-diethyl(trimethylsilyl)amine in dichloromethane was followed by reaction with oxalyl chloride in dichloromethane/*N,N*-dimethyl formamide. The resulting crude phosphonic chloride **10** was added to a solution of dimethyl lithiomethylphosphonate[12] in tetrahydrofuran at -78°C to give **11** as a colourless oil. The appearance of two doublets at δ_P 23.08 and 41.36 ($J_{P,P}$ = 3.66 Hz) in the ^{31}P NMR spectrum firmly established the presence of the protected phosphinylphosphonate moiety. Two-step hydrolysis of triester **11** was accomplished by reaction with trimethylsilyl bromide in the presence of *sym*-collidine, followed by treatment with aqueous N ammonia in

methanol, to give crude **12**. Purification was effected by CHP20P column chromatography, using a linear gradient of acetonitrile in water as the eluent, to give target compound **12** (NH_4^+-form) in 57% yield. The presence of two characteristic doublets at δ_P 17.7 and 32.2 ($J_{P,P}$ = 17.1 Hz) was in complete accordance with the presence of the phosphinylphosphonate function in **12**. In addition, the mass spectrum of **12** (H^+-form) recorded in the positive or negative electrospray mode, showed peaks at *m/z* 599 $[M-H]^-$ and 618 $[M+NH_4]^+$, respectively, thus confirming the molecular weight of 600.

The inhibitory action of the thus obtained diastereoisomeric mixture of **12** on SS was tested in rat liver membrane preparations.[13] For the sake of comparison, the known[3c] FPP-analogue **13**[14], having the same pyrophosphate modification as **12**, was also tested under the same conditions.

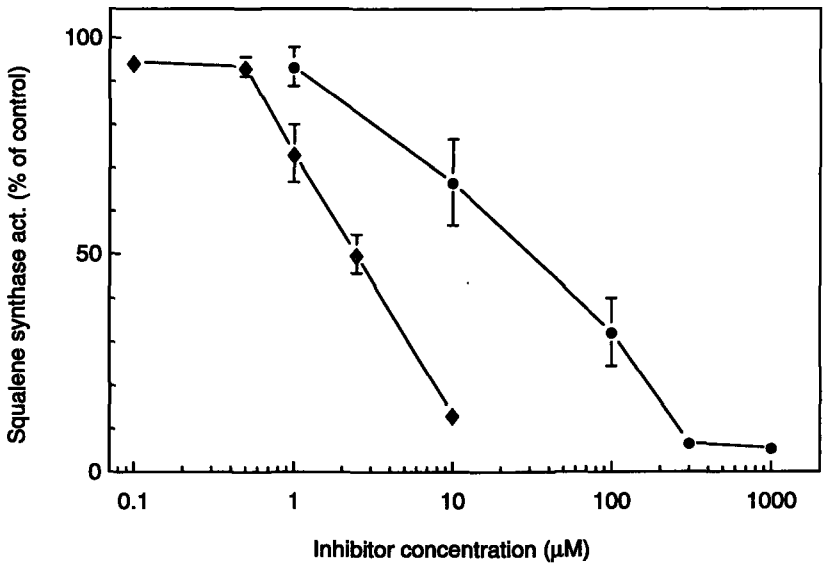


Figure 1. Inhibition of SS activity by compounds 12 and 13.
SS assay was performed in the presence of the indicated concentrations of compound **12** (•) or compound **13** (♦). Values are the mean of three to four separately performed experiments (each experiment consists of duplicate determinations at each concentration) and expressed as percentage of control values (2.15 ± 0.29 mmol squalene formed/min/mg of protein); bars depict S.E.M.[15]

The results, presented in Fig. 1, show that both compounds are inhibitors of SS with IC_{50} values of 38.4 ± 19.9 μM and 2.4 ± 0.3 μM for compound **12** and **13**, respectively. It is evident that the diastereoisomeric mixture **12** is less active than **13**, however, it may not be excluded that only one of the individual stereoisomers of **12** is responsible for the inhibitory effect on SS.

In conclusion, we have presented a high yielding synthesis of an inhibitor of SS, in which the individual steps are easy to perform. The outcome of the enzyme assay justifies the synthesis of the separate isomers of **12** which then have to be tested in different FPP-consuming enzyme assays (*i.e.* SS and protein:farnesyl transferase).

Experimental

General procedures

(E,E)-Farnesol and geranylacetone were purchased from Aldrich and distilled. Toluene, dichloromethane and ether were dried by refluxing with P_2O_5 for 2 hours and then distilled. Toluene and ether were stored over sodium wire. Dichloromethane was stored over molecular sieves (0.4 nm). THF and acetonitrile were dried by refluxing with CaH_2 for 16 h, distilled and stored over molecular sieves (0.4 nm). THF and ether were redistilled from $LiAlH_4$ directly before use. All reactions were carried out under a blanket of argon, unless stated otherwise. TLC-analysis was performed on silicagel (Schleicher & Schull, F 1500 LS 254). Compounds were visualised by spraying the TLC-plates with $KMnO_4$ (1%) in aqueous Na_2CO_3 (2%). Column chromatography was performed on Merck Kieselgel (230-400 Mesh ASTM). Evaporations were carried out below 40°C under reduced pressure (15 mm Hg). ^1H, ^{13}C and ^{31}P NMR spectra were measured at 199.99, 50.1 and 80.7 MHz, respectively, using a JEOL JNM-FX 200 spectrometer on line with a JEC 980 B computer. ^1H, ^{13}C and ^{31}P NMR spectra were recorded using a Bruker WM-300 spectrometer operating at 300, 75 and 121 MHz, respectively. ^1H and ^{13}C chemical shifts are given in ppm (δ) relative to tetramethylsilane (TMS) as internal standard and ^{31}P chemical shifts are given in ppm (δ) relative to 85% H_3PO_4 as external standard.

(E)-6,10-Dimethyl-5,9-undecadiene-2-ol (2)

To a suspension of $LiAlH_4$ (133 mg, 3.5 mmol) in ether (5 mL), was added a solution of geranylacetone (1) (388 mg, 2 mmol) in ether (5 mL). After refluxing for 10 min TLC-analysis (light petroleum/ether 95/5 v/v) showed complete disappearance of the starting compound. The reaction mixture was cooled to 0 °C and dry ethyl acetate (4 mL) was added dropwise, followed by water (4 mL) and 15% NaOH (4 mL). Stirring was continued at 0 °C for 1 h. The organic layer was separated and the remaining salts were washed with ether. The combined organic layers were washed with water, dried over $MgSO_4$ and concentrated. The product was purified by silica gel column chromatography (elution: light petroleum/ether 1/0 → 9/1 v/v) to afford 97% of **2** as a colourless oil.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.7, 17.4 (C12, C13); 23.2 (C1); 24.2, 26.4 (C4, C8); 25.5 (C11); 38.9, 39.5 (C3, C7); 67.4 (C2); 123.8, 124.1 (C5, C9); 131.0, 135.1 (C6, C10);

^1H NMR ($CDCl_3$) δ 1.19 (d, 3 H, H1, $J_{1,2}$ = 6.17 Hz); 1.40-1.55 (m, 2 H, H2); 1.60, 1.63, 1.68 (3xs, 9 H, H11, H12, H13); 1.85-2.09 (m, 6 H, H4, H7, H8); 3.71 (sex, 1H, H2); 5.01-5.15 (m, 2 H, H5, H9).

(E)-6,10-Dimethyl-5,9-undecadiene-2-yl benzenesulfonate (3)

To a cooled (0°C) solution of compound **2** (196 mg, 1 mmol) in dry pyridine (2 mL) was added benzenesulfonyl chloride (160 µl, 1.25 mmol) in dry pyridine (2 mL). After stirring for 16 h at room temperature the reaction mixture was diluted with water and concentrated under reduced pressure. The remainder was dissolved in dichloromethane and washed with water, 10% $NaHCO_3$ and water. The organic layer was dried over $MgSO_4$, concentrated and the residue was applied to a silica gel column, which was eluted with a gradient of light petroleum/ether (1/0 → 1/1 v/v). Concentration of the appropriate fractions gave **3** (yield 74%) as a colourless oil.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.8, 17.5 (C12, C13); 20.7 (C1); 23.3, 26.5 (C4, C8); 25.5 (C11); 36.5, 39.5 (C3, C7); 80.5 (C2); 122.4, 124.0 (C5, C9); 127.5, 129.0, 133.3 (3xC_{arom}); 131.2, 136.1 (C6, C10); 137.5 ($C_{q\text{-}arom}$).
^1H NMR ($CDCl_3$) δ 1.29 (d, 3 H, H1, $J_{1,2}$ = 6.19 Hz); 1.51, 1.59, 1.68 (3xs, 9 H, H11, H12, H13); 1.54-1.64 (m, 2 H, H3); 1.71-2.01 (m, 6 H, H4, H7, H8); 4.65 (sex., 1 H, H2); 4.90-4.96 and 5.01-5.06 (2xm, 2 H, H5, H9); 7.26-7.68 and 7.90-7.96 (2xm, 5 H, H_{arom}).

Diethyl ((E)-1,5,9-trimethyl-4,8-decadienyl)malonate (4)

To a stirred suspension of NaH (60 mg as 80% in oil, 2 mmol) in THF (4 mL) was added diethylmalonate (304 µl, 2 mmol) over a period of 5 min. After stirring for 30 min at RT compound **3** (168 mg, 0.5 mmol) in THF (2 mL) was added and stirring was continued for 4 h at reflux temperature. When TLC analysis (light petroleum/ether 4/1 v/v) showed complete disappearance of **3** the reaction mixture was diluted with ether and washed with saturated NH_4Cl and water. The organic layer was dried over $MgSO_4$, filtrated and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (elution: light petroleum/ether 1/0 → 3/1 v/v) to give **4** as a colourless oil. Yield 79%.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 13.8 ($OCH_2\mathit{C}H_3$); 15.6, 17.3 (C12, C13); 16.5 (C11); 25.3 (C10); 24.9, 26.4 (C3, C7); 32.7 (C1); 34.0 (C2); 39.4 (C7); 57.2 (CH (Malonate)); 60.6 ($O\mathit{C}H_2CH_3$); 123.5, 124.0 (C4, C8); 130.8, 134.9 (C5, C9); 168.3, 168.5 (CO (Malonate)).
^1H NMR ($CDCl_3$) δ 1.00 (d, 3 H, H3', $J_{3,3'}$ = 6.7 Hz); 1.27 (t, 6 H, $OCH_2\mathit{C}H_3$); 1.34-1.55 (m, 2 H, H4); 1.60 (s, 6 H, H7', H11'); 1.68 (s, 3 H, H12); 1.98-2.05 (m, 6 H, H5, H8, H9); 2.19-2.33 (m, 1 H, H3); 3.25 (d, 1 H, H2, $J_{1,2}$ = 7.96 Hz); 4.37 (q, 4 H, $O\mathit{C}H_2CH_3$); 5.05-5.09 (m, 2 H, H6, H10).

Diethyl ((E)-1,5,9-trimethyl-4,8-decadienyl)((E,E)-3,7,11-trimethyl-2,6,10-dodecatrienyl)malonate (5)

To a stirred suspension of NaH (9 mg as 80% in oil, 0.3 mmol) in THF (1 mL) was added a solution of **4** (100 mg, 0.3 mmol) in THF (1 mL). After stirring for 30 min at RT the mixture was treated with a solution of farnesyl bromide (143 mg, 0.5 mmol) in THF (1 mL). After refluxing for for 4 h the reaction mixture was diluted with ether and washed with saturated NH_4Cl and water. The organic layer was dried ($MgSO_4$) and concentrated. Silica gel column chromatography (elution: light petroleum/ether 1/0 → 9/1 v/v) of the crude mixture afforded **5** in a yield of 82%.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 13.9 ($OCH_2\mathit{C}H_3$); 14.5, 15.7, 15.9, 17.3 (C13, C14, C15, C11', C12', C13'); 25.4 (C12, C10'); 26.3, 26.5 (C5, C9, C3', C7'); 32.0 (C1); 32.9 (C2'); 36.3 (C1'); 39.5 (C8, C6'); 39.7 (C4); 60.3 ($O\mathit{C}H_2CH_3$); 61.8 (C_q (Malonate)); 118.7 (C2); 123.8, 124.0, 124.2 (C6, C10, C4', C8'); 130.7 (C11, C9'); 134.6, 134.7 (C7, C5'); 137.4 (C3); 170.6, 170.8 (CO (Malonate));

^{1}H NMR ($CDCl_3$) δ 1.25 (t, 6 H, OCH_2CH_3); 1.60 (s, 15 H, H13, H14, H15, H12', H13'); 1.68 (s, 6 H, H12, H10'); 1.90-2.18 (m, 16 H, H4, H5, H8, H9, H2', H3', H6', H7'); 2.63 (d, 2 H, H1, $J_{3,4}$ = 7.2 Hz); 4.16 (dq, 4 H, OCH_2CH_3); 5.01-5.08 (m, 5 H, H2, H6, H10, H4', H8').

Ethyl (E,E)-2-((E)-1,5,9-trimethyl-4,8-decadienyl)-5,9,13-trimethyl-4,8,12-tetradecatrienoate (6)

To a solution of **5** (1 g, 1.9 mmol) in DMSO (10 mL) was added NaCN (360 mg, 7.4 mmol) and the mixture was heated for 5 h at 180°C. As TLC-analysis (light petroleum/ether 95/5 v/v) showed complete conversion of the starting compound the reaction mixture was cooled to RT and brine was added. This mixture was extracted three times with light petroleum. The combined organic layers were washed with water, dried over $MgSO_4$ and concentrated. The crude compound was purified by silica gel column chromatography (elution: light petroleum/ether 1/0 → 9/1 v/v) to give **6** (73% yield) as an oil.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 14.2 (OCH_2CH_3); 15.7, 15.7, 15.9, 17.3, (C13, C14, C15, C11', C12', C13'); 25.5 (C12, C10'); 26.3, 26.5 (C5, C9, C3', C7'); 32.0 (C1); 32.9 (C2'); 36.3 (C1'); 39.5 (C8, C6'); 39.7 (C4); 60.3 (OCH_2CH_3); 61.8 (C_q (Malonate)); 118.7 (C2); 123.8, 124.0, 124.2 (C6, C10, C4', C8'); 130.7 (C11, C9'); 134.6, 134.7 (C7, C5'); 137.4 (C3); 174.8 (CO (Malonate));

^{1}H NMR ($CDCl_3$) δ 0.85-0.95 (m, 4 H, H1', H11'); 1.11-1.20 (m, 2 H, H2'); 1.24 (t, 3 H, OCH_2CH_3); 1.60 (s, 15 H, H13, H14, H15, H12', H13'); 1.68 (s, 6 H, H12, H10'); 1.91-2.11 (m, 14 H, H4, H5, H8, H9, H3', H6', H7'); 2.17-2.26 (m, 3 H, H2, H3); 4.10 (q, 2 H, OCH_2CH_3); 5.02-5.14 (m, 5 H, H4, H8, H12, H4', H8').

(E,E)-2-((E)-1,5,9-Trimethyl-4,8-decadienyl)-5,9,13-trimethyl-4,8,12-tetradecatriene-1-ol (7)

To a suspension of $LiAlH_4$ (133 mg, 3.5 mmol) in ether (5 mL) was added a solution of **6** (655 mg, 1.4 mmol) in ether (5 mL). After refluxing for 10 min TLC-analysis (light petroleum/ether 95/5 v/v) showed complete disappearance of the starting compound. The reaction mixture was cooled to 0°C and dry ethyl acetate (4 mL) was added dropwise, followed by water (4 mL) and 15% NaOH (4 mL). Stirring was continued at 0°C for 1 h. The organic layer was separated and the remaining salts were washed with ether. The combined organic layers were washed with water, dried over $MgSO_4$ and concentrated. The product was purified by silica gel column chromatography (elution: light petroleum/ether 1/0 → 9/1 v/v) to afford 76% of **7** as a colourless oil.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.5, 15.8, 15.9, 16.1, 17.5 (C13, C14, C15, C11', C12', C13'); 25.5 (C14, C10'); 26.4 (C7, C11, C3', C7'); 31.8 (C3); 32.5, 32.9 (C1'); 34.3 (C2'); 39.6, 39.7 (C6, C10, C6'); 46.0 (C2); 63.5, 64.1 (C1); 123-125 (C4, C8, C12, C4', C8'); 131.0, 134.8, 135.8 (C5, C9, C13, C5', C9').

^{1}H NMR ($CDCl_3$ δ 0.88 (2xd, 3 H, H11'); 1.16-1.26 (m, 2 H, H2, H1'); 1.60 (s, 15 H, H15, H16, H17, H12', H13'); 1.68 (s, 6 H, H14, H10'); 1.92-2.11 (m, 18 H, H3, H6, H7, H10, H11, H2', H3', H6', H7'); 3.56 (d(b), 2 H, H1); 5.01-5.16 (5 H, H4, H8, H12, H4', H8').

Diethyl ([{(E,E)-2-[(E)-1,5,9-trimethyl-4,8-decadienyl]-5,9,13-trimethyl-4,8,12-tetradecatrienyl}oxy]methyl)phosphonate (8)

To a cooled (-78°C) and stirred solution of **7** (429 mg, 1 mmol) in THF (2 mL) under argon was added *n*-BuLi (625 µl as 1.6 M in hexanes, 1 mmol) over 15 min. The reaction was allowed to stir for 40 min at -78°C, when diethyl phosphonomethyl triflate (450 mg, 1.5 mmol) in THF (2 mL) was added. After 30 min at -78°C, the reaction was allowed to warm to 0°C for 2 h. The reaction was quenched with saturated NH_4Cl

and partitioned between ether and water. The ether layer was washed with brine, dried ($MgSO_4$) and evaporated. The crude product was purified by silica gel column chromatography (elution: light petroleum/ether 1/0 → 3/2 v/v) to give homogenous **8** in a yield of 84%.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.1 ($OCH_2\underline{C}H_3$); 16.0, 16.1, 17.2 (C15, C16, C17, C11', C12', C13'); 25.2 (C14, C10'); 26.4 (C7, C11, C3', C7'); 31.7 (C3); 32.2, 32.6 (C1'); 34.0 (C2'); 39.4 (C6, C10, C6'); 43.1 (C2); 61.7, 61.8 ($O\underline{C}H_2CH_3$); 64.8 (d, $O\underline{C}H_2P$, $J_{C,P}$ = 155.6 Hz); 73.8, 74.0 (2xd, C1, $J_{C,P}$ = Hz); 123-125 (C4, C8, C12, C4', C8'); 130.5, 134.3, 135.3 (C5, C9, C13, C5', C9').

^1H NMR ($CDCl_3$) δ 0.85-0.88 (m, 4 H, H1', H11'); 1.34 (t, 6 H, $OCH_2C\underline{H}_3$); 1.60 (s, 15 H, H15, H16, H17, H12', H13'); 1.68 (s, 6 H, H14, H10'); 1.85-2.10 (m, 18 H, H3, H6, H7, H10, H11, H2', H3', H6', H7'); 3.39-3.48 (m, 2 H, H1); 3.73 (d, 2 H, OCH_2P, J = 8.5 Hz); 4.16 (dq, 4 H, $OC\underline{H}_2CH_3$); 5.01-5.12 (m, 5 H, H4, H8, H12, H4', H8').

$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 21.8

Ethyl ([{(E,E)-2-[(E)-1,5,9-trimethyl-4,8-decadienyl]-5,9,13-trimethyl-4,8,12-tetradecatrienyl}oxy]methyl)phosphonate (9)

To a solution of **8** (372 mg, 0.64 mmol) in ethanol (3.2 mL) was added 1 N KOH (3.2 mL), and the reaction was refluxed for 16 h. After cooling to RT, the ethanol was evaporated and the aqueous residue was stirred with dichloromethane and acidified with 10% HCl. The organic layer was washed with water and brine, dried ($MgSO_4$) and evaporated to provide 96% of **9**. This was used without further purification in the next step.

$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 17.4

Dimethyl ({ethyl ([{(E,E)-2-[(E)-1,5,9-trimethyl-4,8-decadienyl]-5,9,13-trimethyl-4,8,12-tetradecatrienyl}oxy]methyl)phosphinyl}methyl)phosphonate (11)

To a stirred solution of monoester **9** (339 mg, 0.62 mmol) in CH_2Cl_2 under argon was added *N,N*-diethyl(trimethylsilyl)amine (220 µl, 1.16 mmol). The reaction was allowed to stir for 1.5 h at RT, the solvent was evaporated and the residue was dissolved in toluene, evaporated and then pumped at high vacuum. The remainder was dissolved in CH_2Cl_2 (2 mL) containing one drop of DMF, under argon at 0°C, and oxalylchloride (107 µl, 1.23 mmol) was added dropwise over a period of 10 min. After 45 min at 0°C and 45 min at RT the solution was evaporated and the residue was twice dissolved in toluene and concentrated to give crude **10**, which was used directly in the next step.

To a solution of dimethyl methylphosphonate (152 µl, 1.4 mmol) in THF (4 mL) at -78°C under argon was added *n*-BuLi (0.85 mL as 1.6 M in hexanes, 1.36 mmol) over 5 min. After 40 min, the acid chloride **10** prepared above was added in THF (2 mL) over 10 min. The reaction was allowed to stir for 1 h at -78°C when it was quenched with saturated NH_4Cl and diluted with ether. The aqueous layer was made acidic with 10% HCl and the organic layer was separated and washed with brine. The aqueous layer was re-extracted with CH_2Cl_2, and the CH_2Cl_2 was washed with brine. The combined organic layers were dried ($MgSO_4$) and evaporated. The crude product was applied to a silica gel column which was eluated with a gradient of CH_2Cl_2/MeOH 1/0 → 9/1 v/v to give **11** as a colourless oil (59% yield).

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.8 ($OCH_2\underline{C}H_3$); 13.6, 16.3, 16.4, 17.5 (C15, C16, C17, C1', C12', C13'); 25.5 (C14, C10'); 25.9, 26.6 (C7, C11, C3', C7'); 26.5 (q, PCH_2P, $J_{C,P}$ = 74.6); 34.0 (C3); 34.3 (C2'); 39.6, 39.7

(C6, C10, C6'); 43.2 (C2); 61.4 (OCH_2CH_3); 66.7 (d, OCH_2P, $J_{C,P}$ = 119.9 Hz); 75.0 (C1); 122.9, 123.0, 124.0, 124.4, 124.9 (C4, C8, C12, C4', C8'); 131.0, 134.6, 134.7, 135.8 (C5, C9, C13, C5', C9').
^1H NMR ($CDCl_3$) δ 0.85-0.88 (m, 4 H, H1', H11'); 1.34 (t, OCH_2CH_3); 1.60 (s, 15 H, H15, H16, H17, H12', H13'); 1.68 (s, 6 H, H14, H10'); 1.85-2.06 (m, 18 H, H3, H6, H7, H10, H11, H2', H3', H6', H7'); 2.36-2.58 (m, 2 H, PCH_2P); 3.40-3.50 (m, OCH_2P); 3.78-3.85 (m, 8 H, OCH_3, OCH_2CH_3); 4.13-4.21 (m, 2 H, H1); 4.99-5.12 (m, 5 H, H4, H8, H12, H4', H8').
^{31}P NMR: δ 23.1, 41.4 (2xd, $J_{P,P}$ = 3.66 Hz)

({([{(E,E)-2-[(E)-1,5,9-trimethyl-4,8-decadienyl]-5,9,13-trimethyl-4,8,12-tetradecatrienyl}oxy]methyl)hydroxyphosphinyl}methyl)phosphonic acid (tri ammonium salt) (12)
To a stirred solution of 11 (240 mg, 0.36 mmol) in CH_2Cl_2 (2 mL) at RT was added 2,4,6-collidine (199 µl, 0.9 mmol) followed by bromotrimethylsilane (237 µl, 1.8 mmol). The reaction was allowed to stir for 23 h at RT, the solution was evaporated, the residue was dissolved in toluene, evaporated and pumped at high vacuum. The remainder was dissolved in 1 M NH_4OH (5 mL) and stirred for 30 min at RT, diluted with water and lyophilized.
$^{13}C\{^1H\}$ NMR (D_2O) δ 15.7, 16.5, 16.6, 17.1, 18,0 (C15, C16, C17, C11', C12', C13'); 26.0 (C14, C10'); 27.5 (C7, C11, C7'); 33.5 (C3); 35.5 (C2'); 40.5 (C6, C10, C6'); 43.6 (C2); 71.5 (OCH_2P, $J_{C,P}$ = 117 Hz); 75.5 (C1); 123.4, 124.5, 125.3, 125.6, 125.7 (C4, C8, C12, C4', C8'); 131.4, 135.3, 136.2, 136.9 (C5, C9, C13, C5', C9').
^1H NMR (D_2O) δ 0.83 and 0.91 (2xd, 3 H, H11', $J_{28,15}$ = 6.1 Hz); 1.55, 1.57, 1.36 (3xs, 24 H, H14, H15, H16, H17, H10', H11', H12', H13'); 1.91-2.11 (m, 18 H, H3, H6, H7, H10, H11, H2', H3', H6', H7'); 3.37-3.63 (m, 4 H, H1 and OCH_2P); 5.07-5.15 (m, 5 H, H4, H8, H12, H4', H8')
$^{31}P\{^1H\}$ NMR (D_2O) δ 17.7, 32.2 (2xd, $J_{P,P}$=17.1 Hz)

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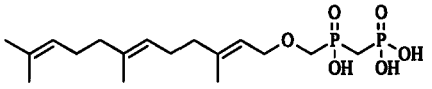
14  **13**

15 S.E.M. = Standard Error of Mean.

CHAPTER 4

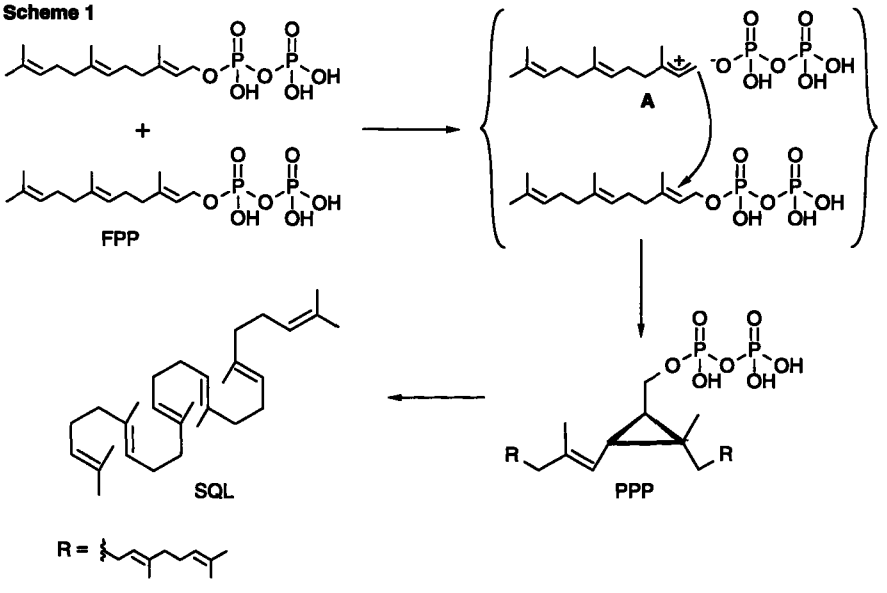
Synthesis of Pyrophosphonic Acid Analogues of Farnesyl Pyrophosphate[1]

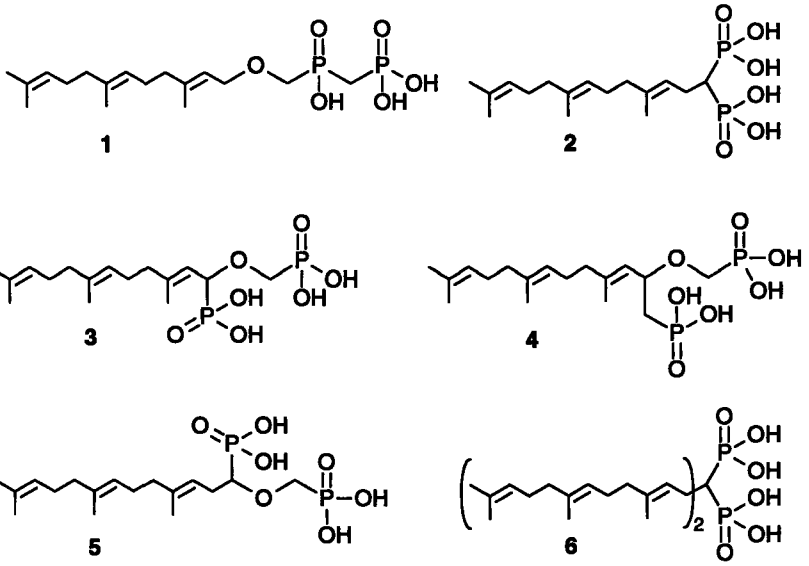
Abstract: The synthesis of four new analogues (*i.e.* **3**-**6**) of farnesyl pyrophosphate (FPP), which may function as inhibitor of squalene synthase, is described. Compounds **3** and **4** were readily accessible by reaction of farnesal with diethyl phosphite or dimethyl lithiomethylphosphonate, respectively, followed by condensation of the resulting alcohols with diethyl phosphonomethyl triflate. The preparation of **5** and **6** was accomplished by alkylation of bis(diethyl phosphonomethyl) ether or tetraethyl methylenebisphosphonate, respectively, with farnesyl bromide.

Introduction

The enzyme squalene synthase (SS) catalyses (see Scheme 1) the head-to-head condensation of two farnesyl pyrophosphate (FPP) molecules to give squalene (SQL) *via* the intermediate presqualene pyrophosphate (PPP). The SS-mediated reductive dimerization of two FPP-units, which is the first biosynthetic step leading exclusively to sterols, presents

Scheme 1





an attractive target for inhibition of the cholesterol biosynthesis. Thusfar, several mechanisms have been proposed[2] for the enzymic conversion of FPP into SQL. Recently, Mookhtiar *et al.*[3] postulated that sequential occupation of the donor and acceptor sites of SS by two individual FPP molecules is followed by cleavage of the carbon-oxygen bond of the donor FPP to give an intimate ion pair (see **A** in Scheme 1) of the allylic carbocation and inorganic pyrophosphate. In the next step, an insertion reaction takes place between the C1 of the cation and the C2-C3 double bond of the acceptor FPP to produce PPP, which is further processed to SQL.

The first potent inhibitor of SS (*i.e.* **1**) was reported by Biller *et al.*[4] and contains an oxy-methylphosphinylmethylphosphonate moiety instead of the naturally occurring pyrophosphate function. The allylic oxygen in this analogue is believed to be involved in binding to the donor-site of SS. Later on, Ciosek *et al.*[5] showed that 1,1-bisphosphonate analogues of FPP (*e.g.* compound **2**) are even more potent inhibitors of SS. The latter class of compounds is supposed to mimic, as proposed by Mookhtiar *et al.*,[3] the tight-ion pair of the allylic cation and the inorganic pyrophosphate formed along the reaction pathway from FPP to PPP.

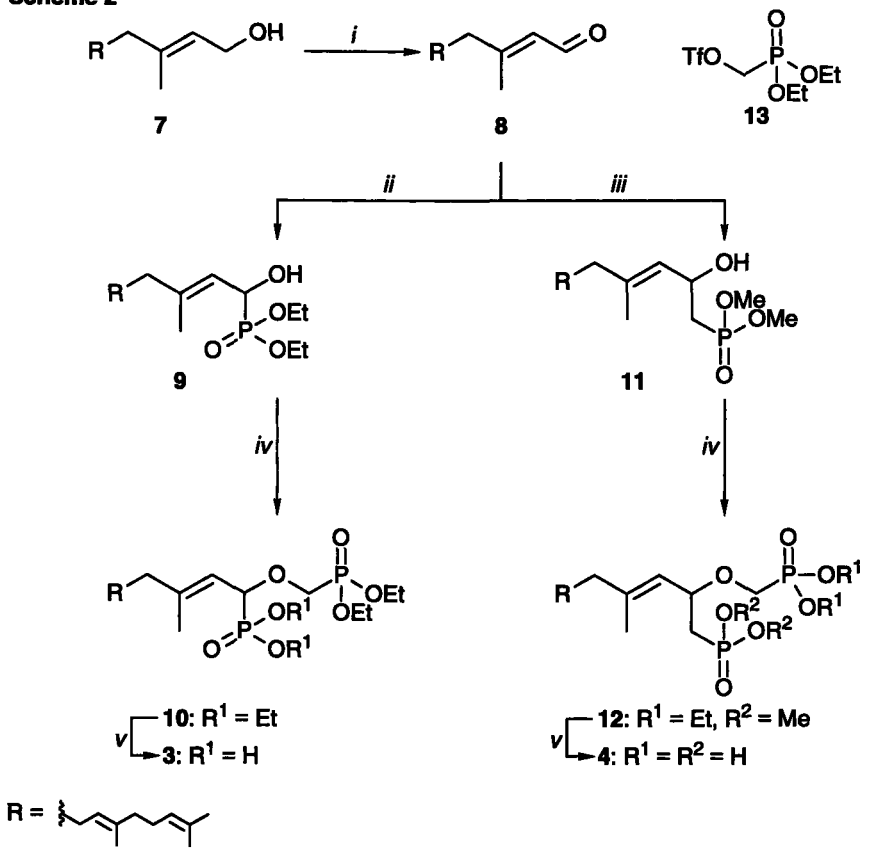
As part of an ongoing[6] program directed towards the design and synthesis of SS-inhibitors, we here report the preparation of four new analogues of FPP (*i.e.* **3**, **4**, **5** and **6**) containing the same phosphonic acid structural elements as the earlier mentioned 1,1-bisphosphonic acid inhibitors. Apart from this, compounds **3-5** also contain an allylic

(as in **3** and **4**) or homoallylic (as in **5**) oxygen, either of which seems to be essential for binding to SS.[4]

Results and Discussion

Retrosynthetic analysis reveals (see Scheme 2) that farnesal (**8**) is an appropriate starting compound in the synthesis of target compounds **3** and **4**. Aldehyde **8** was obtained in nearly quantitative yield by Swern oxidation of farnesol (**7**). The synthesis route to **3** proceeded further with the preparation of α-hydroxy farnesylphosphonate **9**. Initially,

Scheme 2[a]

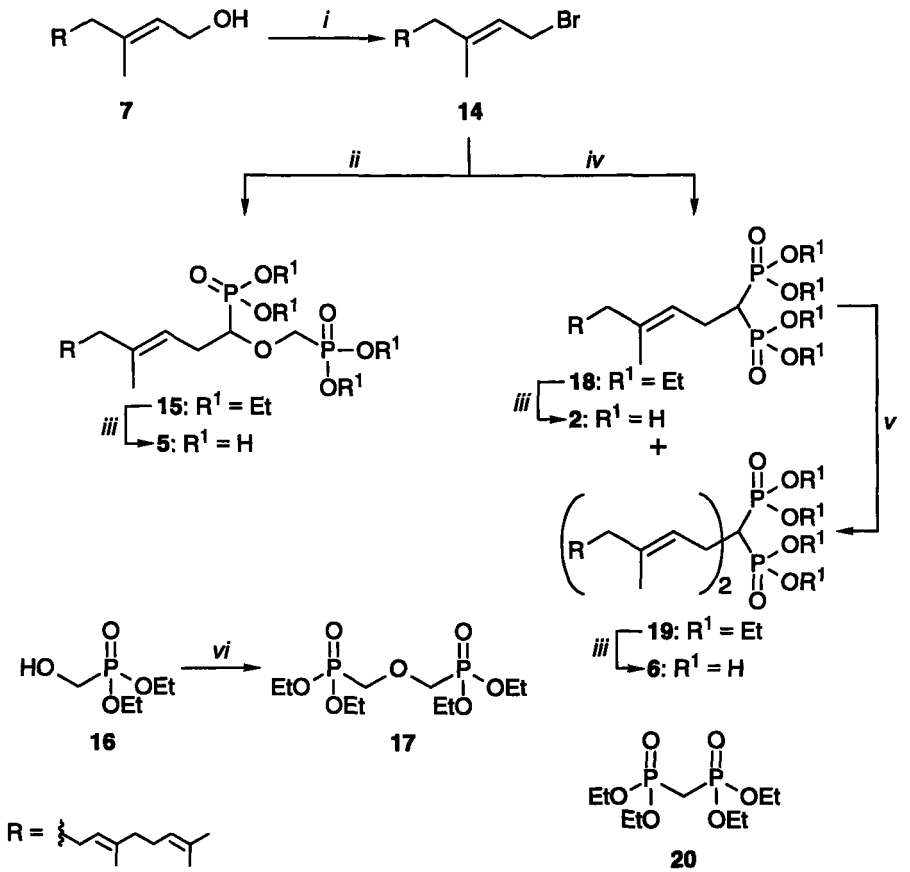


[a] **Reagents and conditions**

i: Swern oxydation; *ii*: $HP(O)(OEt)_2$, NEt_3; *iii*: $LiCH_2P(O)(OMe)_2$, THF, -78 to 0°C; *iv*: *n*-BuLi, 2) **13**, THF, -78 to 0°C; *v*: 1) TMS-Br, *sym*-collidine, CH_2Cl_2, 2) N KOH.

treatment of **8** with diethyl phosphite in diethyl ether and sodium hydride, led to an intractable mixture of products. Fortunately, **9** could be synthesized in 50% yield according to the procedure of Pompliano *et al.*.[7] In the following step, protected bisphosphonate **10** was readily accessible by addition of diethyl phosphonomethyltriflate (**13**)[8] to the lithium alcoholate of **9**, generated *in situ* with *n*-butyllithium in tetrahydrofuran at -78°C. Removal of the phosphonate protecting groups of **10** occurred smoothly by the following two-step procedure. Thus, reaction of **10** with trimethylsilyl bromide (TMS-Br) in the presence of the acid scavenger *sym*-collidine, and subsequent basic hydrolysis of the resulting TMS-ester

Scheme 3[a]



[a]**Reagents and conditions**

i: NBS, dimethyl sulfide, CH_2Cl_2, -40 to 0°C; *ii*: **17**, LDA, THF, -78°C; *iii*: 1) TMS-Br, *sym*-collidine, CH_2Cl_2, 2) N KOH; *iv*: **20**, NaH, THF; *v*: NaH, **14**, THF; *vi*: *n*-BuLi, **13**, THF, -78°C.

with aqueous KOH, yielded crude bisphosphonic acid **3**. Purification of the crude reaction mixture by CHP20P-gel chromatography[9] afforded homogeneous **3** (K^+-salt) in 65% yield.

A similar route was followed to the synthesis of FPP-analogue **4**. Thus, condensation of farnesal **8** with dimethyl lithiomethylphosphonate gave the requisite β-hydroxy phosphonate **11** in 75% yield. Transformation of **11** into **4** was achieved by the same sequence of reactions as mentioned earlier for the conversion of **9**→**3**. Hence, condensation of the lithium salt of **11** with triflate **13** furnished bisphosphonate **12**. Transesterification of **12**, followed by saponification of the resulting TMS-ester with N KOH provided bisphosphonic acid **4** in a satisfactory yield.

In principle, analogue **5** could be prepared in a similar fashion. However, the latter would entail a three-step homologation sequence of farnesal to homofarnesal[10] prior to the introduction of the two phosphonic acids. A more direct route to compound **5** is delineated in Scheme 3 and comprises alkylation of bis(diethyl phosphonomethyl) ether (**17**) with farnesyl bromide (**14**). Reagent **17**, previously prepared[11] by nucleophilic displacement of the bromine atoms in bis(bromomethyl) ether with the potassium anion of diethyl phosphite, was readily accessible by treatment of **13** with the lithium salt of diethyl hydroxymethylphosphonate (**16**). Addition of farnesyl bromide (**14**) to the lithiated species of **17** gave protected analogue **15** in 48% yield. The low yield of the alkylation reaction may be ascribed to the destabilizing effect of the neighbouring ether-oxygen on the carbanion of **17**. Deprotection of **15** to give **5** proceeded analogous to the transformation of **10**→**3**.

In order to assess the biological activity of the new FPP-analogues **3**-**5** with a known inhibitor of Ciosek *et al.*,[5] we prepared farnesylmethylbisphosphonic acid **2** starting from farnesyl bromide (**14**)[12] and commercially available tetraethyl methylenebisphosphonate (**20**). Thus, alkylation of **20** with **14** in the presence of sodium hydride, gave major **18** and minor **19** (10%), as gauged by ^{31}P-NMR spectroscopy of the crude reaction mixture. In this respect, it is interesting to note that Sulsky *et al.*[13] reported that alkylation of **20** with allylic halides yielded mainly the disubstituted products. Further processing of **18**, as described earlier, resulted in the isolation of **2** in good yield. At this stage, we reasoned that the disubstituted product **6** may also function as potential inhibitor of SS. Preparation of bisfarnesylmethylenebisphosphonic acid **6** was readily accomplished by alkylation of **18** with bromide **14** in the presence of sodium hydride to afford **19**. Deblocking of **19** proceeded smoothly to give, after purification, homogeneous **6**.

In conclusion, the results presented in this paper show that four new analogues of FPP were readily accessible by a simple and straightforward methodology. These analogues are valuable tools to get a better insight into the structure-activity relationship between FPP-analogues and the enzyme squalene synthase.

Experimental

General procedures

(E,E)-Farnesol was purchased from Aldrich and distilled. Toluene, dichloromethane and ether were dried by refluxing with P_2O_5 for 2 hours and then distilled. Toluene and ether were stored over sodium wire. Dichloromethane was stored over molecular sieves (0.4 nm). THF and acetonitrile were dried by refluxing with CaH_2 for 16 h, distilled and stored over molecular sieves (0.4 nm). THF and ether were redistilled from $LiAlH_4$ directly before use. All reactions were carried out under a blanket of argon, unless stated otherwise. TLC-analysis was performed on silicagel (Schleicher & Schull, F 1500 LS 254). Compounds were visualised by spraying the TLC-plates with $KMnO_4$ (1%) in aqueous Na_2CO_3 (2%). Column chromatography was performed on Merck Kieselgel (230-400 Mesh ASTM). Evaporations were carried out below 40°C under reduced pressure (15 mm Hg). ^1H, ^{13}C and ^{31}P NMR spectra were measured at 199.99, 50.1 and 80.7 MHz, respectively, using a JEOL JNM-FX 200 spectrometer on line with a JEC 980 B computer. ^1H, ^{13}C and ^{31}P NMR spectra were recorded using a Bruker WM-300 spectrometer operating at 300, 75 and 121 MHz, respectively. ^1H and ^{13}C chemical shifts are given in ppm (δ) relative to tetramethylsilane (TMS) as internal standard and ^{31}P chemical shifts are given in ppm (δ) relative to 85% H_3PO_4 as external standard.

(E,E)-Diethyl 1-hydroxy-farnesylphosphonate (9)

To a solution of (E,E)-farnesal (**8**) (1 g, 4.5 mmol) in acetonitrile (5 mL) were added triethylamine (1.25 mL) and diethylphosphite (0.9 mL, 7 mmol). After stirring for 5 days TLC-analysis (ethyl acetate/acetone 9/1 v/v) indicated that no further conversion of farnesal into the target compound took place and the reaction mixture was taken up in ether and washed with water. The organic layer was dried ($MgSO_4$) and evaporated to dryness. Purification of the crude product was effected by chromatography over silica gel (elution: ethyl acetate/acetone 1/0 → 9/1 v/v) to give the title compound as a colourless oil in 50% yield.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.5 (C14); 16.1 (OCH_2CH_3); 16.6 (C13); 17.2 (C15); 25.2 (C12); 26.1 (C5); 26.5 (C9); 39.5 (C4 and C8); 62.2 (OCH_2CH_3); 65.4 (C1, $J_{C,P}$ = 165.3 Hz); 120.4 (C2); 123.5 (C6); 124.1 (C10); 140.4 (C3, $J_{C,P}$ = 14.6 Hz); 134.8 (C7); 130.6 (C11).

^1H NMR ($CDCl_3$): δ 1.26 (t, 6 H, OCH_2CH_3); 1.59, 1.68, 1.72 (3xs, 12 H, H12, H13, H14, H15); 1.98-2.24 (m, 8 H, H4, H5, H8, H9); 4.07-4.23 (m, 5 H, H1, OCH_2CH_3); 5.02-5.15 and 5.26-5.32 (2xm, 3 H, H2, H6, H10).

$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 23.8.

(E,E)-Diethyl 1-(diethyl phosphonomethoxy)-farnesylphosphonate (10)

A cooled (-78°C) solution of **9** (358 mg, 1 mmol) in THF (5 mL) was treated with *n*-BuLi (625 µL, 1.6M in hexane, 1 mmol) and stirring was continued for 40 min. Then diethyl phosphonomethyltriflate (330 mg, 1 mmol) in THF (1 mL) was added and the reaction mixture was stirred for 30 min at -78°C and 2h at 0°C. The reaction was diluted with ether (10 mL), washed with saturated NH_4Cl (5 mL) and dried over $MgSO_4$. Concentration of the organic layer under reduced pressure and purification of the residue by silica gel chromatography (elution: ethyl acetate/acetone 1/0 → 9/1 v/v) gave 95% of the title compound.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.6 (C14); 16.1 (OCH_2CH_3); 16.7 (C13); 17.3 (C15); 25.3 (C12); 25.9 (C5); 26.4 (C9); 39.4 (C8); 39.5 (C4); 62.0 (OCH_2CH_3 and OCH_2P, $^1J_{C,P}$ = 165.3 Hz, $^3J_{C,P}$ = 14.6 Hz); 74.0 (C1, $^1J_{C,P}$

= 172.6 Hz, $^3J_{C,P}$ = 10.2 Hz); 123.9 (C2); 125.2 (C6); 125.6 (C10); 145.6 (C3, $J_{C,P}$ = 13.1 Hz); 137.2 (C7); 135.2 (C11).
$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 20.4, 21.4.

(E,E)-Dimethyl 2-hydroxy-4,8,12-trimethyl-3,7,11-tridecatrienylphosphonate (11)
A cooled (-78°C) solution of dimethyl methylphosphonate (0.46 mL, 4.2 mmol) in THF (15 mL) was treated with *n*-BuLi (2.6 mL, 1.6M in hexane, 4.16 mmol). After 40 min farnesal (440 mg, 2 mmol) in THF (5 mL) was added dropwise and stirring was continued until TLC analysis showed complete disappearance of the starting material. The reaction was quenched with saturated NH_4Cl and diluted with ether. The organic layer was washed twice with water and dried over $MgSO_4$. After evaporation of the solvent the crude material was purified by column chromatography over silica gel (elution: ethyl acetate/methanol 100/0 → 95/5 v/v) to give the title compound in 75% yield.
$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.5 (C15); 16.1 (C14); 17.1 (C16); 25.2 (C13); 25.8 (C6); 26.2 (C10); 32.9 (C1, $J_{C,P}$ = 136.3 Hz); 39.0 (C9); 39.2 (C5); 51.8 (OCH_3); 62.8 (C2, $J_{C,P}$ = 2.9 Hz); 123.3 (C7); 123.8 (C11); 126.7 (C3, $J_{C,P}$ = 14.7 Hz); 131.7 (C12); 134.7 (C8); 137.5 (C4).
^{31}P NMR ($CDCl_3$) δ 32.5.

(E,E)-Dimethyl 2-(diethyl phosphonomethoxy)-4,8,12-trimethyl-3,7,11-tridecatrienyl-phosphonate (12)
A cooled (-78°C) solution of **11** (512 mg, 1.5 mmol) in THF (5 mL) was treated with *n*-BuLi (0.94 mL, 1.6M in hexane, 1.5 mmol) and stirred for 40 min. Then diethyl phosphonomethyltriflate (480 mg, 1.5 mmol) in THF (1 mL) was added dropwise. The reaction mixture was stirred for 30 min at -78°C followed by 2 h at 0°C. The reaction was quenched with saturated NH_4Cl and the mixture was diluted with ether and washed with water. The organic layer was dried over $MgSO_4$ and concentrated. Purification of the crude reaction mixture by silica gel chromatography (elution: ether/acetone 1/0 → 9/1 v/v) afforded 80% of the title compound.
$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 14.5 (OCH_2CH_3); 15.7 (C15); 16.0 (C14); 17.1 (C16); 24.9 (C13); 25.5 (C6); 26.0 (C10); 30.8 (C1, $J_{C,P}$ = 139.2 Hz); 39.0 (C9); 39.0 (C5); 51.6 (OCH_3); 60.2 (OCH_2P, $J_{C,P}$ = 170.0 Hz); 62.1 (OCH_2CH_3); 72.0 (C2, $J_{C,P}$ = 14.6 Hz); 122.6 (C7); 122.8 (C11); 122.5 (C3, $J_{C,P}$ = 11.7 Hz); 130.3 (C12); 134.7 (C8); 142.0 (C4).
^{31}P NMR ($CDCl_3$) δ 31.5, 22.7.

(E,E)-Diethyl 1-(diethyl phosphonomethoxy)-4,8,12-trimethyl-3,7,11-tridecatrienyl-phosphonate (15)
A cooled (-78°C) solution of bis(diethyl phosphonomethyl) ether (600 mg, 2 mmol) in THF (2 mL) was treated with LDA (2 mmol) and stirred for 30 min. Then farnesyl bromide (1.2 mmol) in THF (2 mL) was added and stirring was continued for another hour. When TLC analysis showed no further conversion of the starting compound the reaction was quenched by addition of saturated NH_4Cl. The mixture was diluted with ether and the organic layer was washed with saturated NH_4Cl and brine, dried over $MgSO_4$ and concentrated. Purification of the crude oil over silica gel (elution: ethyl acetate/acetone 1/0 → 9/1 v/v) yielded 48% of the title compound as a colourless oil.
$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.8 (C15); 15.9 (C14); 16.0 (OCH_2CH_3); 16.1 (C16); 25.2 (C13); 26.1 (C6); 26.3 (C10); 28.6 (C2); 39.3 (C9); 39.4 (C5); 62.0 (OCH_2CH_3); 65.1 (OCH_2P, $J_{C,P}$ = 165.3 Hz); 77.9 (C1, $J_{C,P}$ =

160.9 and 13.1 Hz); 118.7 (C3, $J_{C,P}$ = 11.7 Hz); 123.6 (C7); 123.9 (C11); 130.7 (C12); 134.6 (C8); 137.6 (C4).
^{1}H NMR ($CDCl_3$) δ 1.34 (t, 12 H, OCH_2CH_3); 1.60, 1.64, 1.68 (3xs, 12 H, H13, H14, H15, H16); 1.93-2.16 (m, 8 H, H5, H6, H9, H10); 2.40-2.53 (m, 2 H, H2); 3.61-3.72 (m, 1 H, H1); 5.10-5.33 (m, 3 H, H3, H7, H11).
$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 20.6, 22.3

Bis(diethyl phosphonomethyl) ether (17)
A cooled (-78°C) solution of diethyl hydroxymethylphosphonate (840 mg, 5 mmol) in THF (5 mL) was treated with *n*-BuLi (1.6 M, 3.1 mL, 5 mmol) and the reactionmixture was stirred for 40 min at -78°C. Then diethyl phosphonomethyltriflate (1.5 g, 5 mmol) in THF (5 mL) was added and the reaction mixture was allowed to warm to 0°C in 2 h. The reaction was stopped by addition of saturated NH_4Cl, the mixture was diluted with ether, washed with brine and dried over $MgSO_4$. After removal of the solvent the crude oil was purified by silica gel chromatography (elution: ethyl acetate/acetone 1/0 → 9/1 v/v) to give 69% of **17**.
$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 16.2 (OCH_2CH_3); 60.1 (dd, OCH_2P, $^1J_{C,P}$ = 164 Hz, $^3J_{C,P}$ = 15 Hz); 62.3 (OCH_2CH_3);
$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 20.0.
Anal. calcd. for $C_{10}H_{24}O_7P_2$: P 19.5; found P 19.65%

(E,E)-Tetraethyl farnesylmethylene-1,1-bisphosphonate (18)
To a cooled (0°C) suspension of NaH (67 mg, 2.8 mmol) in THF (2 mL) was added dropwise a solution of tetraethylmethylene bisphosphonate (746 µL, 3 mmol) in THF (2 mL). After stirring for 30 min farnesyl bromide (2 mmol) in THF (2 mL) was added and stirring was continued for 2 hours. When TLC analysis showed complete conversion of the starting material, the reaction was stopped by addition of ethanol. The reaction mixture was diluted with ether and washed with saturated NH_4Cl and brine and dried over $MgSO_4$. Purification of the crude mixture by silica gel chromatography (elutoin: ethyl acetate/acetone 1/0 → 9/1 v/v) afforded pure **18** in 62% yield.
$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.3 (C15); 15.6 (C14); 15.7 (OCH_2CH_3); 17.2 (C16); 23.4 (t, C2, $J_{C,P}$ = 4.4 Hz); 24.9 (C13); 25.8 (C10); 26.0 (C6); 36.9 (t, C1, $J_{C,P}$ = 134 Hz); 39.0 (C5 and C9); 121.3 (t, C3, $J_{C,P}$ = 7.3 Hz); 123.3 (C7); 123.7 (C11); 130.2 (C12); 134.1 (C8); 135.8 (C4).
$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 24.2.

(E,E,E,E)-Tetraethyl bis(farnesyl)methylene-1,1-bisphosphonate (19)
To a cooled (0°C) suspension of NaH (240 mg, 10 mmol) in THF (5 mL) was added dropwise a solution of compound **15** (4 mmol) in THF (5 mL). After stirring for 30 min farnesyl bromide (5 mmol) in THF (5 mL) was added and stirring was continued for 2 hours. When TLC analysis showed complete conversion of the starting material, the reaction was stopped by addition of ethanol. The reaction mixture was diluted with ether and washed with saturated NH_4Cl and brine and dried over $MgSO_4$. Purification of the crude mixture by silica gel chromatography (elution: ethyl acetate/acetone 1/0 → 9/1 v/v) afforded homogeneous **19** in 64% yield.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.2, 15.6, 15.9 (C14, C15, C16); 15.8 (OCH_2CH_3); 24.9 (C13); 25.9, 26.0 (C6, C10); 28.5 (C2); 39.0, 39.4 (C5, C9); 45.2 (t, C1, $J_{C,P}$ = 130 Hz); 118.7 (C3); 123.5, 123.7 (C7, C11); 130.1 (C12); 134.0 (C8); 136.1 (C4).
^1H NMR ($CDCl_3$) δ 1.32 (t, 12 H, OCH_2CH_3); 1.59, 1.62, 1.68 (3xs, 24 H, H13, H14, H15, H16); 1.99-2.17 (m, 16 H, H5, H6, H9, H10); 2.60 (dt(b), 4 H, H2); 4.17 (quin., 8H, OCH_2CH_3); 5.01-5.19 and 5.31-5.47 (2xm, 6 H, H3, H7, H11).
$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 27.0.

General procedure for the deprotection of compounds 10, 12, 15, 18 and 19

The starting material (1 mmol) was dissolved in a mixture of dichloromethane (5 mL) and *sym*-collidine (0.7 mL, 5 mmol). Then TMS-Br (0.85 mL, 6.25 mmol) was added and the reaction mixture was stirred for 16 h at RT. The volatiles were removed by evaporation and the residue was treated with aqueous KOH (0.5M, 10 mL). The mixture was stirred for 30 min at RT and then concentrated under reduced pressure. The crude compound was purified on a CHP20P column that was eluted with a linear gradient of 80% acetonitrile/water in 5% methanol/water. Lyophilization of the appropriate fractions yielded the deprotected compounds **2-6**.

2: Yield: 68%. $^{13}C\{^1H\}$ NMR (D_2O) δ 16.2 (C14, C15); 18.0 (C16); 25.0 (C2); 25.9 (C13); 27.0, 27.3 (C6, C10); 40.0, 40.2 (C5, C9); 40.8 (t, C1, $J_{C,P}$ = 112 Hz); 125.1 (C3); 125.5 (C7, C11); 133.0 (C12); 136.5 (C8); 136.6 (C4).
^1H NMR (D_2O) δ 1.56, 1.59, 1.64 (3xs, 12 H, H13, H14, H15, H16); 1.77 (tt, 1 H, H1, $J_{H,P}$ = 21 Hz, $J_{1,2}$ = 7 Hz); 1.93-2.10 (m, 8 H, H5, H6, H9, H10); 2.47 (tt, 2 H, H2, $J_{1,2} = J_{2,3}$ = 6.9 Hz, $J_{H,P}$ = 15.4 Hz); 5.10 (t, 1 H, H3, $J_{2,3}$ = 6.8 Hz); 5.18, 5.47 (2xt, 2 H, H7, H11).
$^{31}P\{^1H\}$ NMR (D_2O) δ 20.7.

3: Yield: 65%. $^{13}C\{^1H\}$ NMR (D_2O) δ 16.1 (C14); 17.1 (C13); 17.9 (C15); 25.8 (C12); 26.8 (C5); 27.0 (C9); 39.8 (C8); 40.3 (C4); 121.0 (C2); 125.2 (C6); 125.4 (C10); 133.6 (C11); 136.9 (C7); 143.7 (C3, $J_{C,P}$ = 9 Hz).
^1H NMR (D_2O) δ 1.58, 1.60, 1.65, 1.71 (4xs, 12 H, H12, H13,H14, H15); 1.95-2.19, (m, 8 H, H4, H5, H8, H9); 3.11-3.26 (m (b), 1 H, H_A OCH_2P); 3.56-3.69 (m (b), 1 H, H_B OCH_2P); 4.07-4.18 (m (b), 1 H, H1); 5.11-5.21 (m, 3 H, H2, H6, H10).
$^{31}P\{^1H\}$ NMR (D_2O) δ 16.1, 17.0.
Anal. calcd. for $C_{16}H_{30}O_7P_2$: P 15.6; found P 15.49%

4: Yield: 63%. $^{13}C\{^1H\}$ NMR (D_2O) δ 16.4 (C15); 17.0 (C14); 18.1 (C16); 26.1 (C13); 27.1 (C6); 27.2 (C10); 35.6 (C1, $J_{C,P}$ = 130.0 Hz); 40.2 (C9); 40.2 (C5); 65.3 (OCH_2P, $J_{C,P}$ = 155.0 Hz); 75.5 (C2); 124.9 (C7); 125.2 (C11); 125.7 (C3, $J_{C,P}$ = 15.3 Hz); 132.4 (C12); 136.3 (C8); 141.0 (C4).
^1H NMR (D_2O) δ 1.58 (s, 3 H, H16); 1.59 (s, 3 H, H15); 1.65 (s, 3 H, H13); 1.72 (s, 3 H, H14); 1.95-2.12 (m, 8 H, H5, H6, H9, H10); 3.34 (ABX, 1 H, $H_A(OCH_2P)$, $J_{H,P}$ = 8.6 Hz, J_{AB} = 13.2 Hz); 3.60 (ABX, 1 H, $H_B(OCH_2P)$, $J_{H,P}$ = 7.5 Hz, J_{BA} = 13.2 Hz); 4.54 (q, 1 H, H2); 5.08-5.14 (m, 3 H, H3, H7, H11).
^{31}P NMR (D_2O) δ 20.79, 15.93.
Anal. calcd. for $C_{17}H_{32}O_7P_2$: P 15.1; found P 14.94%

5: Yield: 70%. $^{13}C\{^1H\}$ NMR (D_2O) δ 16.2 (C15); 16.5 (C14); 17.9 (C16); 25.9 (C13); 26.9 (C6); 27.2 (C10); 30.6 (C2); 39.9 (C9); 40.1 (C5); 70.7 (OCH_2P, $J_{C,P}$ = 149.6 and 7.0 Hz); 82.5 (C1, $J_{C,P}$ = 152.5 and 13.7 Hz); 124.1 (C3, $J_{C,P}$ = 11.5 Hz); 125.2 (C7); 125.6 (C11); 133.7 (C12); 136.9 (C8); 137.4 (C4).
^1H NMR (D_2O) δ 1.64, 1.65, 1.71 (3xs, 12 H, H13, H14, H15, H16); 2.01-2.17 (m, 8 H, H5, H6, H9, H10); 2.40 and 2.55-2.60 (6 lines and m, 2 H, H2); 3.31 (dt, 1 H, H1, $J_{H,P}$ = 4.0 Hz, $J_{1,2}$ = 8.8 Hz); 3.64 (ABX, 2

H, OCH_2P, J_{A-B} = 11.6 Hz, $J_{H,P}$ = 9.2 Hz); 5.19, (t (b), 1 H, H7, $J_{6,7}$ = 7.0 Hz); 5.25 (t (b), 1 H, H11, $J_{10,11}$ = 6.3 Hz); 5.51 (t (b), 1 H, H3, $J_{2,3}$ = 6.8 Hz);
$^{31}P\{^1H\}$ NMR (D_2O) δ 15.0, 17.6.
Anal. calcd. for $C_{17}H_{32}O_7P_2$: P 15.1; found P 15.17%
6: Yield: 67%. $^{13}C\{^1H\}$ NMR (D_2O) δ 16.4, 16.7, 18.1 (C14, C15, C16); 26.1 (C13); 27.5, 27.6 (C6, C10); 28.5 (C2); 40.4, 40.7 (C5, C9); 122.2 (C3); 125.3, 125.5 (C7, C11); 131.7 (C12); 135.7 (C8); 136.6 (C4).
^1H NMR ($CDCl_3$) δ 1.57, 1.60, 1.64, 1.65 (4xs, 24 H, H13, H14, H15, H16); 1.95-2.10 (m, 16 H, H5, H6, H9, H10); 2.58-2.66 (m, 4 H, H2); 5.09, 5.17, 5.57 (3xt(b), 6 H, H3, H7, H11).
$^{31}P\{^1H\}$ NMR (D_2O) δ 25.8.
Anal. calcd. for $C_{31}H_{55}O_6P_2$: P 10.6; found P 10.49%

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CHAPTER 5

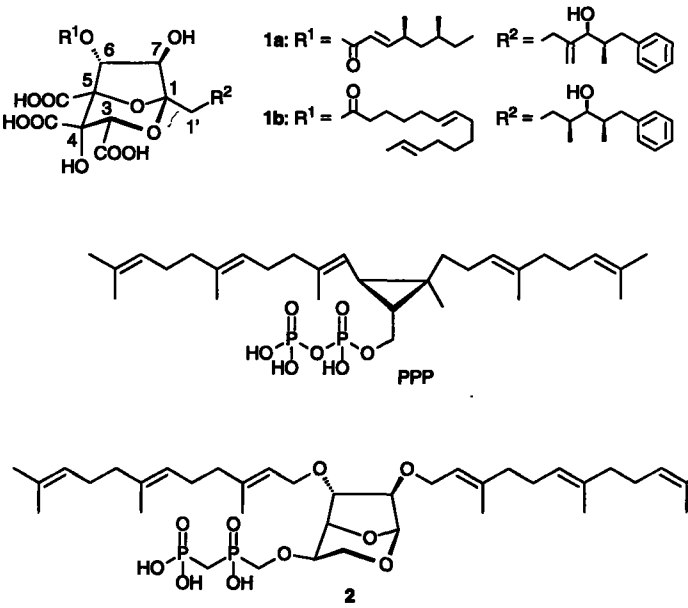
Synthesis of 1,6-Anhydro-2,3-di-*O*-farnesyl-5-*O*-([{phosphonomethyl}phosphinyl]methyl)-α-D-galactofuranose: A Zaragozic Acid - Presqualene Pyrophosphate Hybrid[1]

Abstract: The synthesis of a new potential inhibitor (*i.e.* compound **2**) of squalene synthase is described. This compound is a hybrid of presqualene pyrophopshate, an intermediate in the enzymic conversion of farnesyl pyrophosphate to squalene, and the recently discovered zaragozic acids, which are picomolar competitive inhibitors of squalene synthase.

Introduction

Recently, researchers at Glaxo Group Research Ltd elucidated[2] the structure of three novel fungal metabolites, the so-called squalestatins 1-3, isolated [3] from the culture *Phoma* sp. C2932. Apart from this, it was also established[4] that these compounds are potent picomolar competitive inhibitors of squalene synthase. The latter enzyme catalyses the head-to-head condensation of two farnesyl pyrophosphate molecules to squalene at the final branch-point of the cholesterol biosynthetic pathway. At the same time, researchers at Merck Research Laboratories[5-7] isolated the structurally related zaragozic acids A-C from the fungal cultures *Sporormiella intermedia* (zaragozic acid A) and *Leptodontium elatius* (zaragozic acids B and C). The common structural element in this new class of fungal metabolites is the polar 2,8-dioxabicyclo[3.2.1]octane-4,6,7-trihydroxy-3,4,5-tricarboxylic acid core. On the other hand, they are distinguished from each other by the presence of different acyl and alkyl side-chains at O6 and C1 (see for instance the structure of squalestatin 2 (**1a**) and zaragozic acid B (**1b**) in Fig. 1). Recently, Dufresne *et al.*[6] postulated that the highly effective inhibitory action of the zaragozic acids (squalestatins) on squalene synthase may be ascribed to their topological similarity (*i.e.* both molecules possess rigid triacid cores flanked by two lipophilic residues) with presqualene pyrophosphate (PPP), an intermediate in the squalene synthase-mediated conversion of farnesyl pyrophosphate to squalene. The structural similarity between PPP and zaragozic acids (squalestatins) goaded us to design a hybrid of **1** and PPP, containing a rigid core, two

Figure 1



lipophilic farnesyl residues and a triacid phosphinylphosphonate moiety.

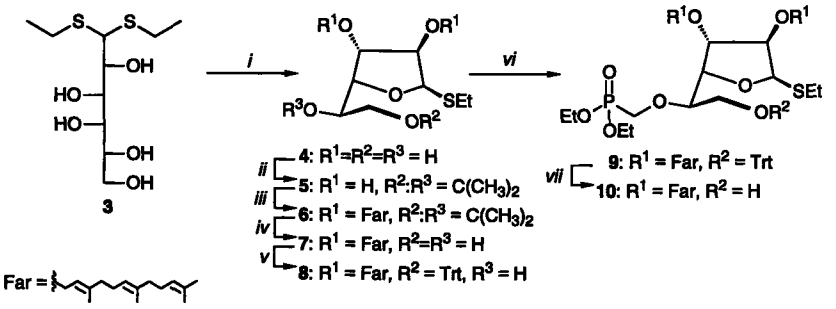
As part of an ongoing program[8] to synthesize effective squalene synthase inhibitors we here report the preparation of compound **2**, the 4,6,7-trihydroxy-2,8-dioxabicyclo[3.2.1]-octane core of which is substituted with two farnesyl moieties at O6 and O7 and a phosphonomethylphosphinylmethyl function at O4.

Results and discussion

Retrosynthetic analysis of target compound **2** reveals that D-galactose is an appropriate starting material for the preparation of the bicyclic core. Initially, the synthesis of compound **2** was pursued by the sequence of reactions outlined in Scheme 1, the key step of which entails cyclisation of 1-ethyl 2,3-di-*O*-farnesyl-5-*O*-(diethyl phosphonomethyl)-1-thio-α-D-galactofuranoside **10** to the corresponding 1,6-anhydro derivative **16** (see Scheme 2).

The synthetic pathway to the key intermediate **10** commences with the acid mediated cyclisation[9] of the readily available[10] D-galactose diethyl dithioacetal (**3**) to give the ethyl 1-thio-α-D-galactofuranoside (**4**). Selective acetonation of the 5,6-diol in **4** with 2,2-dimethoxypropane in DMF and a catalytic amount of camphorsulphonic acid gave partially protected **5**. Bis-farnesylation of **5** with farnesyl chloride[11] in DMF and sodium hydride was unfruitful. However, the required di-farnesylated product **6** could be isolated

Scheme 1[a]

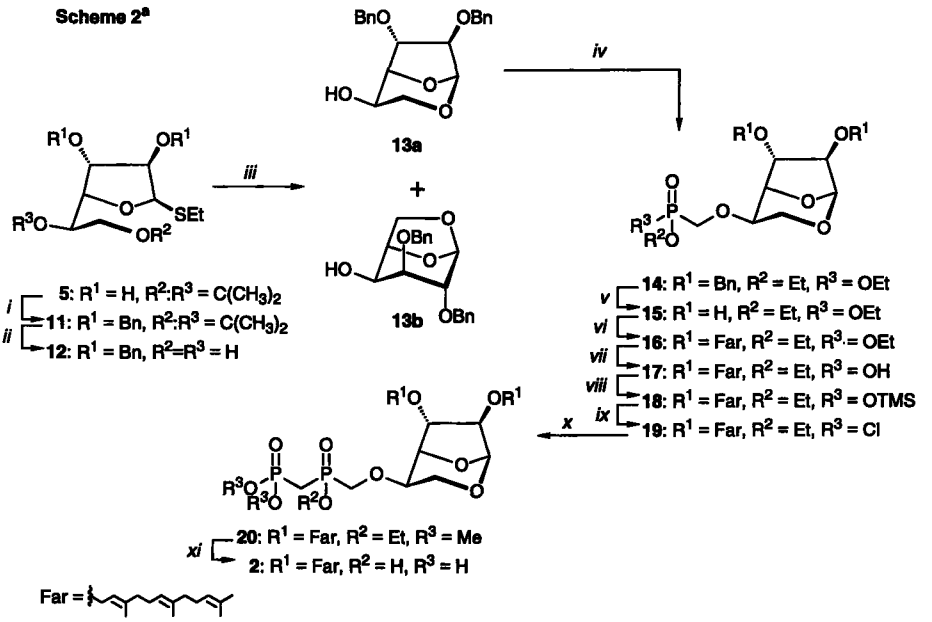


[a]**Reagents and conditions**

i: 1) dil. HCl, 2) HgO (72%); *ii*: 2,2-dimethoxypropane, DMF, cat. camphorsulfonic acid (70%); *iii*: Farnesyl bromide, NaH, DMF (44%); *iv*: $CuCl_2$, *iso*-propanol (71%); *v*: Trityl chloride, pyridine, 65°C (85%); *vi*: KH, THF, $TfOCH_2P(O)(OEt)_2$ (52%); *vii*: HCOOH, MeOH, 0°C (100%).

in 44% yield using farnesyl bromide[12] as the alkylating agent. Unfortunately, the effectivity of the farnesylation could not be improved. Deacetonation of **6** could be effected with $CuCl_2.2H_2O$ in *iso*-propanol[12] to give **7** in 71% yield. In this respect it is of interest to note that deblocking of acetonide **6** could not be realized using aqueous acetic acid. Further, employment of pyridinium *p*-toluene sulfonate as the proton donor gave diol **7** in a moderate yield of 50%. The unexpected stability of the isopropylidene function under these conditions may be due to the lipophilic nature of the farnesyl residues. Treatment of **7** with trityl chloride in pyridine at elevated temperature, followed by phosphonylation of **8** with diethyl phosphonomethyl triflate[13] and potassium hydride, afforded the fully protected galactofuranoside derivative **9** in an overall yield of 44%. Acidolysis of **9** proceeded quantitatively to give the requisite intermediate **10**. Bromonium-ion (NBS) mediated cyclisation of **10** led to complete recovery of the starting material. Moreover, cyclisation of **10** using $FeCl_3$ in acetonitrile, as reported earlier by Åberg *et al.*,[14] was also unsuccessful. The failure to cyclisize **10** may be ascribed to the presence of the lipophilic farnesyl residues, which shield the anomeric thioethyl group in the armed[15] galactofuranoside derivative from electrophilic attack.

It occurred to us that introduction of the farnesyl residues at a later stage of the synthesis would circumvent the problems mentioned above. To this end, we first prepared compound **12**, as delineated in Scheme 2. Hence, benzylation of **5** and subsequent deacetonation of **11** with aqueous acetic acid furnished diol **12**. Cyclisation of benzylated **12** according to the procedure of Åberg *et al.*[14] gave the expected 1,6-anhydro-furanoside **13a** and 1,6-anhydro-pyranoside **13b** in a ratio of 9 to 1. Purification of the reaction mixture by silica gel column chromatography afforded pure **13a** in 64% yield. The ^{13}C and ^{1}H NMR spectroscopical data of **13a** were in complete accordance with those of the same



[a]Reagents and conditions

i: Benzyl bromide, NaH, DMF (85%); *ii*: 80% HOAc (91%); *iii*: $FeCl_3$, CH_3CN, reflux (**13a**: 64%, **13b**: 7%); *iv*: KH, THF, $TfOCH_2P(O)(OEt)_2$ (68%); *v*: H_2, $Pd(OH)_2$ (100%); *vi*: Farnesyl bromide, NaH, DMF (40%); *vii*: N KOH/ethanol, reflux; *viii*: *N,N*-Et_2NTMS; *ix*: oxalyl chloride/DMF, 0°C; *x*: $LiCH_2P(O)(OMe)_2$, THF, -78°C (41%); *xi*: 1) TMS-Br, *sym*-collidine, 2) N KOH (35%).

compound prepared starting from ethyl 1-thio-2,3-di-*O*-benzyl-α-D-galactopyranoside. Phosphonylation of **13a** with diethyl phosphonomethyl triflate led to the fully protected derivative **14**. Hydrogenolysis of **14** in the presence of $Pd(OH)_2$ and consecutive farnesylation of crude diol **15** with farnesyl bromide and sodium hydride furnished farnesylated compound **16** in 40% yield. Saponification of diester **16** and subsequent silylation[16] of the intermediate mono-ester **17** was followed by conversion of resulting **18** into phosphonic chloride **19**. The latter was treated with dimethyl lithiomethylphosphonate, obtained *in situ* by reaction of dimethyl methylphosphonate with *n*-butyllithium at -78°C, to produce protected phosphinylphosphonate **20** in 41% yield over the four steps. The presence of the phosphinylphosphonate function in **20** was unambiguously ascertained by ^1H, ^{13}C and ^{31}P NMR spectroscopy. Finally, the phosphorus protecting groups were removed by transesterification of **20** with trimethylsilyl bromide and basic hydrolysis of the resulting TMS-esters to furnish target compound **2**. The ^1H, ^{13}C and ^{31}P NMR data of **2** were in complete accordance with the proposed structure.

In conclusion, we have shown that compound **2**, a hybrid of presqualene pyrophosphate and zaragozic acid, is readily accessible by a straightforward route, starting from D-galactose. The inhibitory activity of **2** on the enzyme squalene synthase is presently

under investigation. The outcome of the enzyme-assay may provide better insight in the viability of this type of compounds as inhibitors of squalene synthase.

Experimental

General procedures

(E,E)-Farnesol was purchased from Aldrich and distilled. Pyridine was dried by refluxing with CaH_2 for 16 h and then distilled, redistilled from *p*-toluenesulfonyl chloride (60 g/l), redistilled from KOH (40 g/l) and stored over molecular sieves (0.4 nm). Toluene, dichloromethane and ether were dried by refluxing with P_2O_5 for 2 hours and then distilled. Toluene and ether were stored over sodium wire. Dichloromethane was stored over molecular sieves (0.4 nm). THF and acetonitrile were dried by refluxing with CaH_2 for 16 h, distilled and stored over molecular sieves (0.4 nm). THF and ether were redistilled from $LiAlH_4$ directly before use. TLC-analysis was performed on silicagel (Schleicher & Schull, F 1500 LS 254). Compounds were visualised by spraying the TLC-plates with $KMnO_4$ (1%) in aqueous Na_2CO_3 (2%) and by charring with conc. sulfuric acid/methanol (2/8 v/v). Column chromatography was performed on Merck Kieselgel (230-400 Mesh ASTM). Evaporations were carried out below 40°C under reduced pressure (15 mm Hg). ^1H, ^{13}C and ^{31}P NMR spectra were measured at 199.99, 50.1 and 80.7 MHz, respectively, using a JEOL JNM-FX 200 spectrometer on line with a JEC 980 B computer. ^1H, ^{13}C and ^{31}P NMR spectra were recorded using a Bruker WM-300 spectrometer operating at 300, 75 and 121 MHz, respectively. ^1H and ^{13}C chemical shifts are given in ppm (δ) relative to tetramethylsilane (TMS) as internal standard and ^{31}P chemical shifts are given in ppm (δ) relative to 85% H_3PO_4 as external standard.

D-Galactose diethyl dithioacetal (3).

To a solution of D-galactose (50 g, 277.5 mmol) in concentrated hydrochloric acid (75 mL) was added ethyl mercaptane (50 mL, 675 mmol) under vigorous stirring. After 5 min a little ice and ice-water were added. The reaction mixture solidified immediately and more ice-water was added. The solid was filtered and washed with ice-water. The raw material was crystallized from ethanol and recrystallized from hot water to give 59% of the title compound.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 13.6 (SCH_2CH_3); 23.6, 23.9 (SCH_2CH_3); 54.5 (C1); 63.3 (C6); 69.0, 669.9, 70.1, 70.4 (C2, C3, C4, C5).

Ethyl 1-thio-α-D-galactofuranoside (4).

D-Galactose diethyl dithioacetal (14.3 g, 50 mmol)was dissolved in a solution of water (300 mL) and concentrated hydrochloric acid (4.25 mL). After 20 h of vigorous stirring at RT mercuric oxide (yellow, 16.45 g, 76 mmol) was added and stirring was continued for 5 h. The reaction mixture was cooled to 0°C and filtered off. The clear colourless filtrate was concentrated *in vacuo* and the residue was evaporated 3 times with methanol. Crystalline D-galactose was removed by repeated treatments of the residue with absolute methanol and absolute ethanol at 0°C. The alcoholic solution was evaporated to give the title compound in sufficient pure state for the next step (yield 72%).

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.5 (SCH_2CH_3); 25.6 (SCH_2CH_3); 63.7 (C6); 72.0, 78.0, 78.7, 85.4 (C2, C3, C4, C5); 88.8 (C1).

Ethyl 5,6-*O*-isopropylidene-1-thio-α-D-galactofuranoside (5).

Ethyl 1-thio-α-D-galactofuranoside (5.6 g, 25 mmol) was evaporated with and dissolved in DMF (10 mL) and treated with 2,2-dimethoxypropane (3.4 mL, 27.5 mmol) and camphorsulfonic acid (50 mg). After stirring overnight at RT the reaction mixture was quenched with triethylamine (5 mL) and concentrated under reduced pressure. Column chromatography of the residue (elution: CH_2Cl_2/MeOH 1/0 → 95/5 v/v) afforded pure **5** in a yield of 70%.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.3 (SCH_2CH_3); 25.4 (SCH_2CH_3); 25.6, 26.1 ($C(CH_3)_2$); 65.3 (C6); 76.6, 78.1, 78.3, 83.3 (C2, C3, C4, C5); 88.7 (C1); 110.0 ($C(CH_3)_2$).

^1H NMR ($CDCl_3$) δ 1.32 (t, 3 H, SCH_2CH_3); 1.39, 1.43 (2xs, 6 H, $C(CH_3)_2$); 2.73 (AB, 2 H, SCH_2CH_3); 5.34 (d, 1 H, H1, $J_{1,2}$ = 3.8 Hz).

Ethyl 2,3-di-*O*-benzyl-5:6-*O*-isopropylidene-1-thio-α-D-galactofuranoside (11).

To a stirred solution of ethyl 5,6-*O*-isopropylidene-1-thio-α-D-galactofuranoside (**5**, 2.64 g, 10 mmol) in DMF was added NaH (720 mg, 30 mmol) at 0°C. After stirring for 30 min, benzyl bromide (2.6 mL, 22 mmol) was added and stirring was continued at RT for 2 h. The reaction mixture was quenched with dry methanol and evaporated. The residue was dissolved in ether, washed with H_2O, 10% $NaHCO_3$, H_2O and dried over $MgSO_4$. After evaporation of the solvent the residue was purified by silica gel column chromatography (petroleum ether 40-60/ether 1/0 → 8/2 v/v). Combination of the appropriate fractions gave homogenous **11** in 85% yield.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.1 (SCH_2CH_3); 24.5 (SCH_2CH_3); 25.2, 26.7 ($C(CH_3)_2$); 65.2 (C6); 71.9, 72.4 (2xCH_2 Bn); 77.1, 82.5, 83.4, 84.5 (C2, C3, C4, C5); 86.5 (C1); 109.4 ($C(CH_3)_2$); 127.2-128.5 (C_{arom} Bn); 137.5, 137.7 (2xC_q Bn).

Ethyl 2,3-di-*O*-benzyl-1-thio-α-D-galactofuranoside (12).

Compound **11** (2.2 g, 5 mmol) was dissolved in 80% acetic acid (20 mL) and stirred for 2 h at 75°C. When TLC-analysis (ether) showed that the reaction was complete the solvent was removed *in vacuo* and the residue was evaporated 5 times with toluene (10 mL). Purification of the remaining oil by silica gel column chromatography (elution: ether) gave **12** in 91% yield.

$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 14.6 (SCH_2CH_3); 24.5 (SCH_2CH_3); 63.2 (C6); 71.5, 71.7 (2xCH_2 Bn); 71.2, 81.9, 82.8, 83.4 (C2, C3, C4, C5); 86.5 (C1); 127.9-128.4 (C_{arom} Bn); 136.7, 137.1 (2xC_q Bn).

1,6-Anhydro-2,3-di-*O*-benzyl-α-D-galactofuranose (13a).

Compound **12** (2.02 g, 5 mmol) was dissolved in acetonitrile (375 mL) and anhydrous $FeCl_3$ (227 mg, 1.4 mmol) was added. The reaction mixture was refluxed for 30 min, after which period TLC-analysis (CH_2Cl_2/ether 5/1 v/v) showed complete conversion of the starting material. The solvent was removed and the residue was purified by silica gel column chromatography (elution: CH_2Cl_2/ether 1/0 → 5/1 v/v) to give 64% of **13a** and 7 % of **13b**.

13a: $^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 62.3 (C5); 65.3 (C6); 71.2, 72.3 (2xCH_2 Bn); 80.9, 81.6, 85.2 (C2, C3, C4); 96.7 (C1); 127.8-128.3 (C_{arom} Bn); 137.3, 137.4 (2xC_q Bn).
^1H NMR ($CDCl_3$) δ 3.68 (t, 1 H, H6a, $J_{6a,6b}$ = 10 Hz); 4.01 (ddd, 1 H, H6b); 4.03-4.11 (m, 1 H, H5); 4.14 (dq, 1 H, H2); 4.19 (d, 1 H, H3); 4.23 (d(b), 1 H, H4); 5.30 (d, 1 H, H1, $J_{1,2}$ = 3.8 Hz).

1,6-Anhydro-2,3-di-*O*-benzyl-5-*O*-(diethyl phosphonomethyl)-α-D-galactofuranose (14).
Compound **13a** (342 mg, 1 mmol) was dissolved in DMF (5 mL) and treated with KH (80 mg, 2 mmol). After stirring for 1 h diethyl phosphonomethyl triflate (330 mg, 1.1 mmol) was added and stirring was continued for 5 h. When TLC-analysis (ether) indicated that no further reaction took place the reaction was quenched with methanol, diluted with ether and washed with 10% $NaHCO_3$. The organic layer was dried over $MgSO_4$ and evaporated. Purification of the residue by silica gel column chromatography (elution: ether) yielded compound **14** in 68% yield.
$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 16.2 (OCH_2CH_3); 62.2 (OCH_2CH_3); 63.3 (C6); 63.5 (d, OCH_2P, $J_{C,P}$ = 167.6 Hz); 71.9 (d, C5, $J_{C,P}$ = 10.3 Hz); 71.1, 72.1 (2xCH_2 Bn); 79.0 (C4); 81.1, 85.2 (C2, C3); 96.8 (C1); 127.5-128.1 (C_{arom} Bn); 137.2, 137.4 (2xC_q Bn);
^1H NMR ($CDCl_3$) δ 1.24 (dt, 6 H, 2xOCH_2CH_3); 3.66-3.80 (m, 4 H, H6a, H5, OCH_2P); 3.98-4.10 (m, 7 H, H2, H3, H6b, 2xOCH_2CH_3); 4.34 (d(b), 1 H, H4); 4.39-4.55 (2xAB, 4 H, 2xOCH_2 Bn); 5.25 (d, 1 H, H1, $J_{1,2}$ = 4.36 Hz).
$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 20.6.

1,6-Anhydro-5-*O*-(diethyl phosphonomethyl)-α-D-galactofuranose (15).
To a solution of compound **14** (492 mg, 1 mmol) in *iso*-propanol/water (1/1 v/v, 5 mL) was added $Pd(OH)_2$ (25 mg). The mixture was shaken overnight in a Parr-apparatus under an atmosphere of hydrogen. The $Pd(OH)_2$ was filtered off and the solution was concentrated *in vacuo*. The residue was used without purification in the next step.
$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.8, 15.9 (2xOCH_2CH_3); 62.1, 62.3, 62.4, 62.5 (2xOCH_2CH_3); 62.6 (C6); 62.7 (d, OCH_2P, $J_{C,P}$ = 166.7 Hz); 71.8 (d, C5, $J_{C,P}$ = 10.2 Hz); 75.0, 80.4, 81.6 (C2, C3, C4); 98.1 (C1).
$^{31}P\{^1H\}$ NMR ($CDCl_3$) δ 20.9.

1,6-Anhydro-2,3-di-*O*-farnesyl-5-*O*-(diethyl phosphonomethyl)-α-D-galactofuranose (16).
Crude compound **15** was dissolved in DMF (5 mL) and NaH (96 mg, 4 mmol) was added. After stirring for 1 h, the reaction mixture was treated with farnesyl bromide (1.2 mL, 4 mmol) and stirring was continued until TLC-analysis (MeOH/CH_2Cl_2 2/8 v/v) showed no further conversion of the starting material. The reaction was stopped with MeOH and the mixture was diluted with ether, washed with water, 10% $NaHCO_3$ and water and dried over $MgSO_4$. After evaporation of the solvent the residue was purified by silicagel column chromatography (elution: MeOH/CH_2Cl_2 0/1→1/9 v/v) to give the title compound as a colourless oil in 40% yield.
$^{13}C\{^1H\}$ NMR ($CDCl_3$) δ 15.9 (OCH_2CH_3); 16.4, 16.5, 17.6, 25.7 (8xCH_3 Farnesyl); 26.3, 26.7, 39.6, 39.7 (8xCH_2 Farnesyl); 62.5 (OCH_2CH_3); 63.5 (C6); 63.7 (d, OCH_2P $J_{C,P}$ = 167.9); 72.3 (d, C5, $J_{C,P}$ = 9.6 Hz); 66.1, 66.9 (2xOCH_2 Farnesyl); 79.2, 81.3, 85.3 (C2, C3, C4); 97.3 (C1); 120.1, 123.7, 124.3 (3xCH Farnesyl); 135.3, 140.6, 141.3 (3xC_q Farnesyl).

$^{31}P\{^{1}H\}$ NMR ($CDCl_3$) δ 20.7.

1,6-Anhydro-2,3-di-*O*-farnesyl-5-*O*-(ethyl {[dimethyl phosphonomethyl]phosphinyl}-methyl)-α-D-galactofuranose (20).

To a solution of **16** (300 mg, 0.42 mmol) in ethanol (4 mL) was added 1 N KOH (4 mL), and the reaction was refluxed for 16 h. After cooling to RT, the ethanol was evaporated and the aqueous residue was stirred with dichloromethane and acidified with 10% HCl. The organic layer was washed with water and brine, dried ($MgSO_4$) and evaporated to provide **17**. This was used without further purification in the next step.

To a stirred solution of monoester **17** in CH_2Cl_2 under argon was added *N,N*-diethyl(trimethylsilyl)amine (162 µl, 0.81 mmol). The reaction was allowed to stir for 1.5 h at RT, the solvent was evaporated and the residue was dissolved in toluene (10 mL) and evaporated. The remainder was redissolved in CH_2Cl_2 (2 mL) containing one drop of DMF, under argon at 0 °C, and oxalylchloride (73 µl, 0.84 mmol) was added dropwise. After 45 min at 0 °C and 45 min at RT the solution was evaporated and the residue was twice dissolved in toluene (5 mL) and concentrated to give phosphonic chloride **19**.

To a solution of dimethyl methylphosphonate (100 µl, 0.92 mmol) in THF (2.5 mL) at -78 °C under argon was added *n*-BuLi (560 µl as 1.6 M in hexanes, 0.9 mmol). After 40 min, the acid chloride **19** prepared above was added in THF (2 mL). The reaction was allowed to stir for 1 h at -78 °C before it was quenched with saturated NH_4Cl and diluted with ether. The aqueous layer was made acidic with 10% HCl and the organic layer was separated and washed with brine. The aqueous layer was re-extracted with CH_2Cl_2, and the CH_2Cl_2 was washed with brine. The combined organic layers were dried ($MgSO_4$) and evaporated. The crude product was applied to a silica gel column which was eluated with a gradient of CH_2Cl_2/MeOH 100/0 to 90/10 v/v to give **20** as a colourless oil (41% yield over the four steps).

$^{13}C\{^{1}H\}$ NMR ($CDCl_3$) δ 15.8 (OCH_2CH_3); 16.4, 17.5, 18.0, 25.5 (8xCH_3 Farnesyl); 26.1, 26.5, 39.4, 39.5 (8xCH_2 Farnesyl); 52.9 (OCH_3); 63.3 (OCH_2CH_3); 63.4 (C6); 63.9 (d, OCH_2P $J_{C,P}$ = 163.1); 72.4 (d, C5, $J_{C,P}$ = 14.7 Hz); 66.5, 66.8 (2xOCH_2 Farnesyl); 79.1, 81.2, 85.1 (C2, C3, C4); 97.2 (C1); 120.0, 123.6, 124.1 (3xCH Farnesyl); 135.2, 140.5, 141.3 (3xC_q Farnesyl).

^{1}H NMR ($CDCl_3$) δ 1.33-1.39 (m, 3 H, OCH_2CH_3); 1.59, 1.68, 1.69 (3xs, 24 H, 8xCH_3 Farnesyl); 1.95-2.12 (m, 16 H, 8xCH_2 Farnesyl); 2.40-2.57 (m, 2 H, PCH_2P); 3.58-3.86 (m, 8 H, 2xOCH_3, H5, H6a); 3.88-4.23 (m, 11 H, OCH_2P, 2xOCH_2 Farnesyl, OCH_2CH_3, H2, H3, H6b); 4.35 (t(b), 1 H, H4); 5.07-5.11 and 5.36-5.39 (2xm, 6 H, 6xCH Farnesyl); 5.29 and 5.30 (2xd, 1 H, H1).

$^{31}P\{^{1}H\}$ NMR ($CDCl_3$) δ 22.7, 40.1, 41.2

1,6-Anhydro-2,3-di-O-farnesyl-5-O-({hydroxy[dihydroxy phosphonomethyl]phosphinyl}methyl)-α-D-galactofuranose tripotassium salt (2).

To a stirred solution of **20** (100 mg, 0.125 mmol) in CH_2Cl_2 (2 mL) at RT was added *sym*-collidine (50 µl, 0.38 mmol) followed by bromotrimethylsilane (75 µl, 0.42 mmol). The reaction was allowed to stir for 23 h at RT. The volatiles were removed *in vacuo* and the residue was redissolved in toluene (5 mL) and evaporated. The remainder was treated with 1 N KOH (5 mL) and stirred for 30 min at RT, diluted with water and lyophilized. The crude mixture was purified on a CHP20P column that was eluted with a linear gradient of

80% acetonitrile/water in 5% methanol/water. Lyophilization of the appropriate fractions yielded 35% of pure **2** (tripotassium salt).

Anal. calcd for $C_{38}H_{61}P_2O_{10}K_3$: C 53.26,H 7.18, P 7.24. Found: C 53.32, H 7.12, P 7.19.

$^{13}C\{^1H\}$ NMR (D_2O) δ 16.5, 16.9, 18.1, 26.1 (8xCH_3 Farnesyl); 27.5, 40.4 (8xCH_2 Farnesyl); 64.3 (C6); 64.7 (d, OCH_2P $J_{C,P}$ = 153.7); 66.5, 66.6 (2xOCH_2 Farnesyl); 71.7 (d, C5, $J_{C,P}$ = 11.2 Hz); 80.5, 81.3, 84.8 (C2, C3, C4); 97.6 (C1); 120.5, 124.8, 125.0 (3xCH Farnesyl); 131.2, 135.4, 135.4 (3xC_q Farnesyl).

^1H NMR (D_2O) δ 1.58, 1.59, 1.66, 1.70 (4xs, 24 H, 8xCH_3 Farnesyl); 1.97-2.17 (m, 18 H, 4xCH_2 Farnesyl, PCH_2P); 5.30 (d(b), 1 H, H1).

$^{31}P\{^1H\}$ NMR (D_2O) δ 32.0 ($J_{P,P}$ = 17.1 Hz); 13.8 $J_{P,P}$ = 16.9).

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Samenvatting

Een van de meest voorkomende doodsoorzaken in de westerse wereld kan toegeschreven worden aan hart- en vaatziekten. Het ziektebeeld van patiënten met atherosclerose gaat gepaard met opeenhoping van cholesterol in de wand van de bloedvaten, hetgeen op den duur kan leiden tot verstopping van de aders, en uiteindelijk tot een hart- of herseninfarct. Een belangrijke benadering om het verhoogde cholesterolgehalte te verlagen bestaat uit het remmen van de cholesterol biosynthese in het lichaam. In de biosynthese van cholesterol neemt het enzym squaleen synthase (SS) een unieke positie in en is daarom een geschikt aangrijppunt om de aanmaak van cholesterol te remmen. Het enzym katalyseert de vorming van squaleen (SQL) uit twee moleculen farnesyl pyrofosfaat (FPP). Dit proefschrift beschrijft de bereiding van potentiële remmers van het enzym squaleen synthase.

In de *Inleiding* van dit proefschrift wordt de enzymatische werking van SS nader toegelicht. Er wordt een overzicht gegeven van bekende remmers van dit enzym. Bovendien wordt er aandacht besteed aan eiwit:farnesyl transferase (EFT), een enzym dat eveneens FPP als substraat gebruikt. Analoga van FPP, die de werking van SS onderdrukken, kunnen dientengevolge ook een remmende invloed hebben op EFT.

In *Hoofdstuk 1* wordt de synthese en het gebruik beschreven van een nieuw fosfonylerend reagens dat de synthese van FPP-analoga met een gemodificeerde pyrofosfaat functie mogelijk maakt. In de klassieke bereidingswijze van gemodificeerde pyrofosfaten wordt een fosfonzuur geactiveerd m.b.v. dicyclohexylcarbodiimide (DCC) en morfoline. Het zo verkregen fosfonomorfolidaat wordt vervolgens gekoppeld met fosforzuur hetgeen resulteert in een gemodificeerd pyrofosfaat. Het nieuwe fosfonylerend reagens bevat reeds een morfolido functie waardoor de onaantrekkelijke DCC-stap kan worden vermeden. De toepassing van dit nieuwe reagens wordt geïllustreerd door de succesvolle bereiding van drie potentiële remmers van SS.

Een nieuwe manier om FPP-analoga met een gemodificeerde pyrofosfaat functie te bereiden wordt beschreven in *Hoofdstuk 2*. Kenmerkend voor deze methode is dat de fosfonaat diester door milde base-behandeling kan worden verzeept tot een fosfonaat-monoester die op zijn beurt in het overeenkomstige chloride kan worden omgezet. Reactie van het aldus verkregen chloride met morfoline gevolgd door de verwijdering van de overblijvende fosfonaat ethyl-ester geeft, na reactie met fosforzuur, de gewenste gemodificeerde pyrofosfaten.

Het *Addendum* behandelt de biologische activiteit van de FPP-analoga die beschreven zijn in *Hoofdstuk 1* en 2. Deze verbindingen zijn getest op hun remmende werking van SS

en EFT. Uit de resultaten van deze studie kon geconcludeerd worden dat de enzymen SS en EFT verschillende structuurelementen van het gemeenschappelijke substraat FPP herkennen.

In *Hoofdstuk 3* wordt de bereiding beschreven van een potentiële enzym-specifieke remmer van SS. De synthese-route naar deze verbinding bestaat uit de opbouw van het koolstofskelet en de invoering van de gemodificeerde pyrofosfaat functie. Uitgaande van het commercieel verkrijgbare geranyl aceton kon in zes stappen het koolstof skelet verkregen worden, waarna de gemodificeerde pyrofosfaat functie volgens bekende technieken werd ingevoerd.

Een nieuwe klasse van FPP-analoga, die gekenmerkt worden door een bisfosfonaat eenheid, bleek volgens een recente publikatie uitstekende remmende eigenschappen te hebben op SS. De vier negatieve ladingen lijken een belangrijke rol te spelen in de interactie met de active-site van SS. In *Hoofdstuk 4* wordt de synthese besproken van vijf FPP-analoga die twee phosphonaat groepen bevatten terwijl de afstand tussen de fosfor atomen variabel is.

In *Hoofdstuk 5* wordt de synthese beschreven van een verbinding die als een hybride van de recent geïsoleerde zaragozic acids (squalestatines) en presqualeen pyrofosfaat (PPP) kan worden beschouwd. De remmende werking van de zaragozic acids op SS wordt toegeschreven aan de overeenkomst met PPP, een intermediair van de enzymatische omzetting van FPP naar SQL. Op grond van deze gegevens werd de hybride verbinding ontworpen. Uitgaande van D-galactose kon deze potentiële SS-inhibitor in twaalf stappen gesynthetiseerd worden.

Curriculum Vitae

Na het behalen van het VWO diploma aan het Christelijk Lyceum te Alphen aan den Rijn in 1984 begon ik in september van dat jaar aan de studie Scheikunde aan de Rijksuniversiteit Leiden. Het propadeutisch diploma werd in 1985 behaald. De studie voor het doctoraal examen omvatte als hoofdvak organische chemie (prof. dr. J.H. van Boom). Van september t/m december 1989 was ik als gastmedewerker verbonden aan de University of Virginia (Charlottesville, USA) in de werkgroep van prof. dr. S.M. Hecht. In februari 1990 werd het doctoraal examen met goed gevolg afgelegd. Van maart 1990 tot mei 1994 was ik als assistent in opleiding verbonden aan de Rijksuniversiteit Leiden, waar, met financiële steun van TNO-PG, het in dit proefschrift beschreven onderzoek werd verricht in de werkgroep "Synthese van Biopolymeren" onder leiding van prof. dr. J.H. van Boom en dr. G.A. van der Marel. In augustus 1993 bezocht ik het "VIIth European Carbohydrate Symposium" te Krakau. In oktober 1994 nam ik deel aan de "Workshop Selective Synthesis" in Rocamadour.

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