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CHEMISTRY AND BIOCHEMISTRY OF 1-DESOXYSPHINGANINE 1-PHOSPHONATE (DIHYDROSPHINGOSINE-1-PHOSPHONATE)

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Dedicated to Professor David Shapiro on the occasion of his 70th birthday

The chemical synthesis of labelled 1-desoxy-D,L-sphinganine 1-phosphonate has been elaborated. This compound is an analog of sphinganine 1-phosphate, a naturally occurring intermediate in the biological degradation of long chain bases.

The phosphonate is highly toxic when administered intravenously due to its hemolytic effect. The microsomal sphingosine 1-phosphate lyase(aldolase) cleaves [3-^{3}H] 1-desoxysphinganine 1-phosphonate to [1-^{3}H] hexadecanal and aminoethyl phosphonate like sphinganine 1-phosphate however at a reduced rate. The phosphonate is a competitive inhibitor of the lyase (aldolase). K_i has been determined. The molecular dimensions of the phosphonate have been discussed with reference to the aldolase mechanism and known properties of the enzyme.

I. Introduction

The basic component of the large and structurally rather divergent group of sphingolipids is one of the long-chain bases, the predominant representatives of which are 4*t*-sphingenine (sphingosine), sphinganine (dihydrosphingosine) and 4D-hydroxysphinganine (phytosphingosine). Analytical studies by Carter et al. [1–3] and others [4, 5] and the synthetic achievements of Carter et al. [7], Shapiro et al. [8–11] and Jenny and Grob [12, 13] have established the structure and absolute configuration of the naturally occurring bases as D(+)erythro (≡ 2S, 3R) 2-amino-1,3-dihydroxyalkanes. The biosynthetic pathway on which these two optically active centers are introduced has been elucidated in recent years [14–19].

The importance of the absolute configuration of the long-chain bases with regard to their biological degradation has been elaborated in this laboratory [20].

The initial ATP dependent kinase reaction is rather non-specific. The four D(+) and L(−)threo- and erythro-isomers are phosphorylated. However the subsequent pyridoxal phosphate dependent lyase (aldolase) absolutely requires the 2S, 3R configuration for the cleavage into the respective long-chain aldehyde and phosphorylethanolamine. The aldolase is a SH-enzyme and a mechanism has been defined by which a nucleophilic attack of the SH-group at C 3 of all 1-phosphorylated long-chain bases with D(+)erythro structure including phytosphingosine (4D-hydroxysphinganine) is cleaved.

The studies reported in this paper describe the influence of the phosphoryl group and its molecular dimensions on the aldolase reaction.

The degradation in vitro of 1-desoxysphinganine 1-phosphonate (dihydrosphingosine 1-phosphonate) and sphinganine 1-phosphate (dihydrosphingosine 1-phosphate) has been compared and the kinetics of their degradation in the aldolase reaction studied. Furthermore the chemical synthesis of labelled 1-desoxysphinganine 1-phosphonate is described.

II. Experimental

A. Chemical syntheses

[3-^{3}H] erythro ethyl-2-amino-3-hydroxyoctadecanoate(I) was prepared in quantitative yield as described before [21] and [5-^{3}H] cis-2-phenyl-4-carbethoxy-5-pentadecyl-2-oxazoline *(II)* and its $LiAlH_4$ reduction to [5-^{3}H] cis-2-phenyl-4-hydroxymethyl-5-pentadecyl-2-oxazoline *(III)* according to the established procedure of Shapiro et al. [8–11], mp 99–101°C; R_F = 0.72 in TLC solvent system: $CHCl_3$/CH_3OH 10 : 1. The product proved to be chemically and radiochemically pure. [5-^{3}H] cis-2-phenyl-4-methane sulfonyloxymethyl-5-pentadecyl-2-oxazoline (IV): 100 g (2.58 mmole) of *(III)* were dissolved in 10 ml dry pyridine and 380 mg (3.0 mmole) freshly distilled mesylchloride in 5 ml dry pyridine added dropwise at 0°C with stirring. The reaction mixture was stored at 0°C over night. 50 ml ether and 30 ml water were added the etheral phase washed five times with 20 ml water and dried over $MgSO_4$. The solution was concentrated to dryness and the residue recrystallized once from petroleum ether (30–60°C). Yield: 1.191 g (2.56 mmole; 99% of theory); mp 59–62°C. R_F = 0.3, solvent system $CHCl_3/CH_3OH$ 10 : 1.

[5-^{3}H] cis-2-phenyl-4-iodomethyl-5-pentadecyl-2-oxazoline (V): The pure product (IV) was dissolved in 100 ml dry acetone and added to a solution of 8 g (50 mole) sodium iodide in 100 ml dry acetone and refluxed for 24 h. The reaction mixture was concentrated, 200 ml ether and 200 ml water were added and the etherial solution washed with 5% $Na_2S_2O_3$. 1.184 g of [5-^{3}H] cis-2-phenyl-4-iodomethyl-5-pentadecyl-2-oxazoline (V) were obtained and immediately used for the Michaelis-Arbusov-reaction.

[5-^{3}H] cis-2-phenyl-4-diethyl phosphonomethyl-5-pentadecyl-2-oxazoline (VI): 1.184 g of (V) were stirred in 10 g of freshly distilled triethylphosphate for 16 h at 120°C with exclusion of air moisture. A slow stream of dry nitrogen was bubbled through the reaction mixture in order to eliminate ethyliodide formed in the reaction. Excess triethylphosphate was evaporated at 100°C under high vacuum (< 1 torr). 1.47 g of crude product was separated by silicic acid chromatography with ether. 0.942 g (1.96 mmole) of (VI) was obtained, 76% of theory; mp 49.5–50.5°C.

[3-^{3}H] 2-amino-3-hydroxy-octadecyl 1-phosphonic acid (VII): 45.0 mg (89 μmole) (VI) were dissolved in 3 ml glacial acetic acid and 1 ml 48% HBr added The mixture was stirred in a nitrogen atmosphere for 16 h at 100°C. 10 ml water precipitated (VII) as a voluminous white amorphous insoluble compound. It was sedimented by centrifugation and washed several times with water until bromine free. The residue was dried over P_2O_5, then treated with ether, centrifuged, the ether decanted and the residue dissolved in 2 ml 1% KOH, heated for 1 h at 100°C, filtered and neutralized with acetic acid. The precipitate was washed with water, dried over P_2O_5. (VII) was obtained in 60% yield as white amorphous powder; mp decomp. above 200°C. R_F = 0.5 in solvent system: n-butanol/acetic acid/water 60 : 20 : 20.

Elementary analysis:

C found 59.01%; H found 11.00%; N found 3.83%; P found 8.74%
calc.→ 59.1 %; calc.→ 11.1 %; calc.→ 3.83%; calc.→ 8.47%

All melting points are uncorrected.

IR spectra were recorded with grating infrared spectrophotometer, model 257, Perkin Elmer either in nujol or as film, NMR spectra with 60 MHz, model of Varian A 60D. Solvent deuterochloroform with tetramethylsilane as internal standard.

Mass spectra were recorded with a CH 5 mass-spectrometer Varian MAT, emission current of 300 μA and 70 eV. Temperature of ion source 250°C.

Radioactivity was measured in a Tricarb scintillation Counter Model 3380/544 Packard.

Substances with free amino groups were visualized with ninhydrin spray (0.3 g ninhydrin, 5 ml collidin, 100 ml methanol) and phosphor containing compounds with the reagent described by Dittmer and Lester [22]. Radio thin-layer chromatograms were registered with a radiochromatogram scanner Packard, model 7211 or a Berthold scanner model LB 2722.

Periodate oxidation was carried out according to Sweeley and Moscatelli [23], phosphor determination according to Rouser et al. [24] and the cell fractionation following established procedures [25]. Tetrahymena pyriformis were grown in the medium described by Smith et al. [26]. Lipids were extracted by refluxing the tissue with 50 volumes of chloroform/methanol 2 : 1 and 1 : 2 for 15 min, the combined extracts were washed with water, filtered and taken to dryness. The residue was stored under benzene in a nitrogen atmosphere at −20°C.

The incubation mixtures of 0.05 m phosphate buffer pH 7.4 of the in vitro studies contained in a total volume of 2 ml : 1 ml of the respective cell fraction, 0.2 μmol [3-^{3}H] disodium salt of 1-desoxysphinganine 1-phosphonate or -phosphate, 0.1 μmol pyridoxal phosphate and 2 μmol mercaptoethanol. Incubation: 2 h at 37°C. The incubation was stopped with chloroform/methanol 2 : 1 (v/v) and extracted twice. The extract was concentrated to dryness and dissolved in 1 ml dichloroethane. This solution was chromatographed on 1 g of silicic acid in a Pasteur pipette. [1-^{3}H] hexadecanal was completely eluted with 10 ml dichlorethane. The

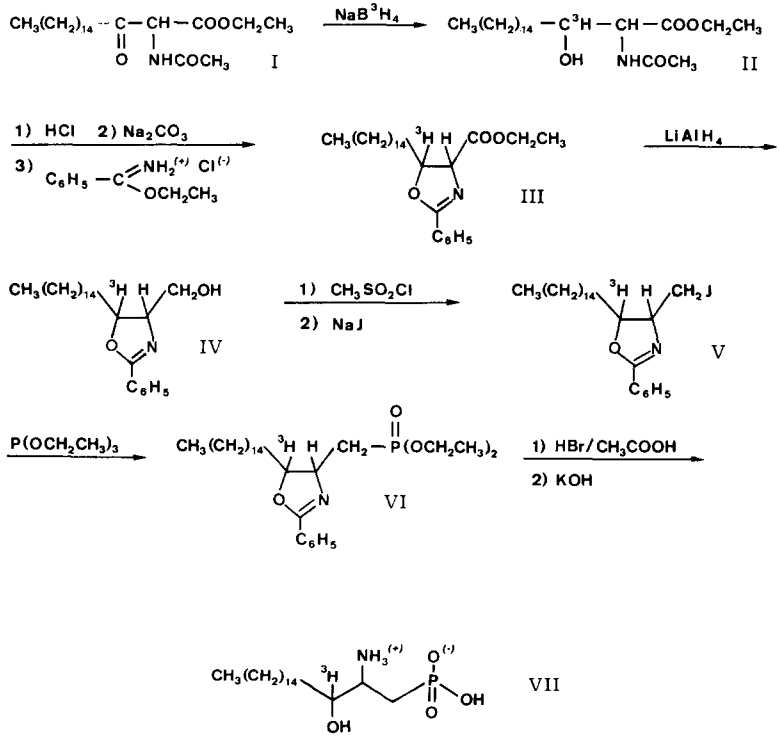
eluate was analyzed by radio thin-layer chromatography for [1-^{3}H] hexadecanal. Solvent system 1.2 dichlorethane: $R_F = 0.5$.

III. Results

A. Chemical synthesis of 1-desoxy sphinganine 1-phosphonate

The biochemical studies described in this paper required the labelled substrate 1-desoxysphinganine 1-phosphonate. Scheme 1 outlines the synthetic route, on which this compound has been labelled at C 3 with tritium.

NaB^3H_4-reduction of erythro ethyl-N-acetyl-2-amino-3-keto-octadecanoate (I) an intermediate in the synthesis of sphinganine [8] yielded erythro [2-^{3}H] ethyl 2-N-acetylamino-3-hydroxyoctadecanoate (II) which after acid hydrolysis of the amide bond in absolute ethanol yielded [3-^{3}H] ethyl 2-amino-3-hydroxyoctadecanoate hydrochloride. Its free base was condensed with ethyl benzimidate hydrochloride to yield [5-^{3}H] cis 2-phenyl-4-carbethoxy-5-pentadecyl oxazoline (III).



Scheme 1.

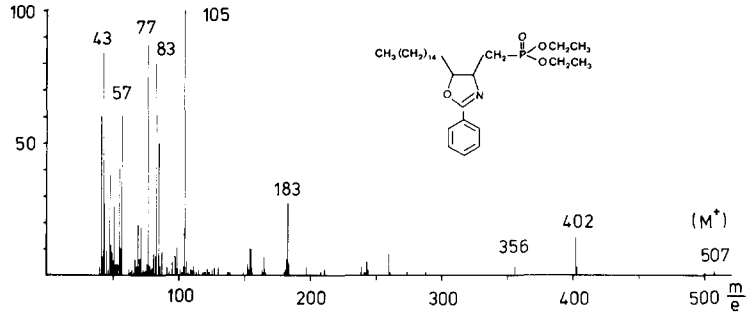


Fig. 1. Mass-spectrum of cis-2-phenyl-4-diethylphosphonomethyl-5-pentadecyl-2-oxazoline.

Reduction with $LiAlH_4$ yielded hydroxy methyl derivative (IV), which was reacted with mesylchloride to the crystalline mesylate.

Exchange of the mesyl group against iodide provided the cis-2-phenyl-4-iodomethyl-5-pentadecyl-2-oxazoline (V). The two most common reactions for the synthesis of phosphonic acid derivatives are the Nylen Reaction [27–29] (reaction of the iodide with sodium diethylphosphite) and Michaelis–Arbusov reaction [27–31], in which iodide is reacted with triethylphosphite.

Although the Nylen reaction has been reported to give good yields also with long chain molecules [24] our long chain substituted iodide reacted most favorably under the conditions of the Michaelis–Arbusov reaction to cis-2-phenyl-4-diethylphosphonomethyl-5-pentadecyl-2-oxazoline. This product could easily be purified by column chromatography and characterized by mass spectroscopy (fig. 1) and ^{1}H-NMR (fig. 2). The molecule ion (M^+–507) and typical fragments are obtained: *m/e* 402 M–105 ($C_6H_5\dot{C}{=}O$) *m/e* 356 M–151 ($\cdot CH_2$–PO $(OC_2H_5)_2$; *m/e* 105 ($C_6H_5\dot{C}{\equiv}O^+$) and 77 ($C_6H_5^+$).

The assignment of proton resonances and their integrals are as follows:

0.8–1.7 δ multiplet of 37 protons. 31 of alkane chain, 6 of which are methylprotons of two ethyl groups; 2.2 δ quartet 2 methylenprotons; J_{H-C-CH}: 7 Hz; and J_{H-C-P}: 19 Hz; 4.2 octet, 4 methylenprotons of ethylgroups J_{H-C-CH}: 7 Hz; J_{HC-O-P}: 12 Hz; 4.6–4.9 δ non resolved multiplet, 2 protons of oxazoline ring; 7.4–8.2 δ multiplet, 5 protons of phenyl residue.

The phosphonic acid diester hydrolysis could be achieved in HBr–acetic acid without byproducts and in high yield. This is essential because the 1-desoxysphinganine 1-phosphonate (VII) is insoluble in organic solvents and water except in acetic acid and as disodium salt and therefore hard to purify by conventional chromatographic procedures.

The end product proved to be chemically and radiochemically pure as shown by (a) elementary analysis (see sect. II), (b) radio thin-layer chromatography (fig. 3), (c) gas chromatography of trimethylsilyl derivative (fig. 4), (d) radio gas chromatography (fig. 5a) and radio thin-layer chromatography (fig. 5b) of [1-^{3}H] hexadecanal released from 1-desoxy sphinganine 1-phosphonate by periodate oxidation, (e)

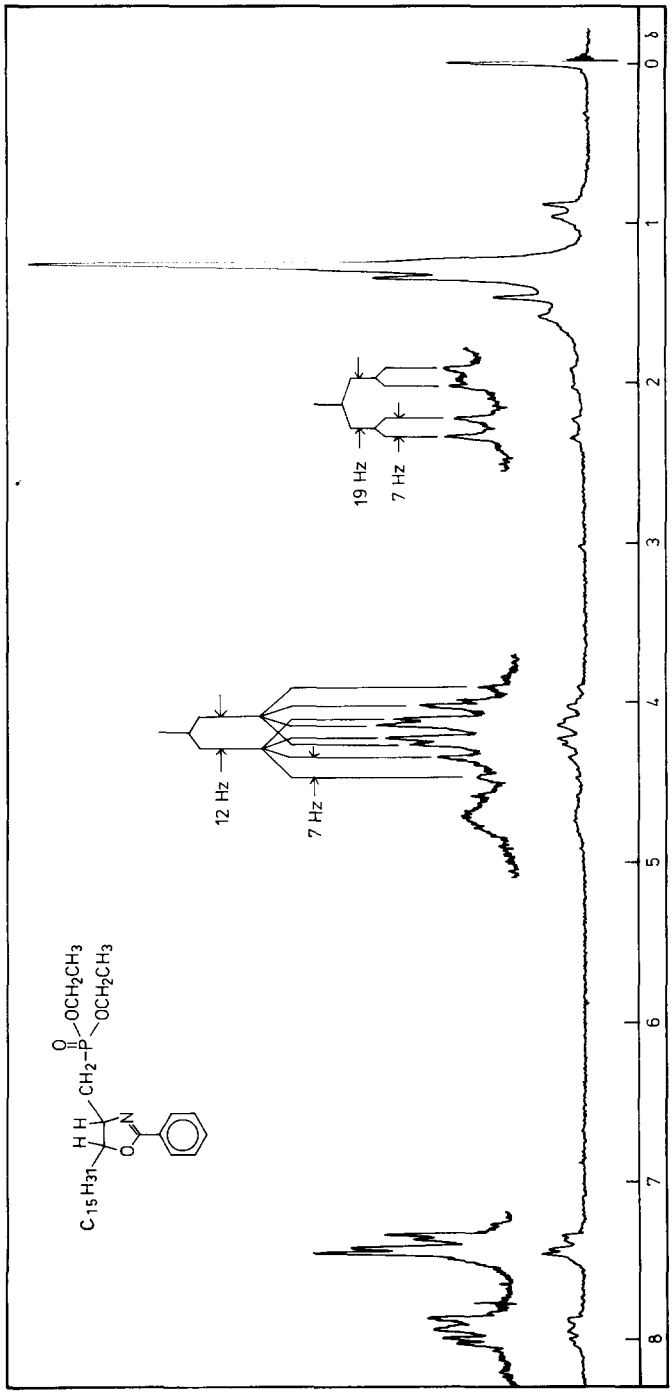


Fig. 2. ^{1}H-NMR-spectrum of cis-2-phenyl-4-diethylphosphonomethyl-5-pentadecyl-2-oxazoline.

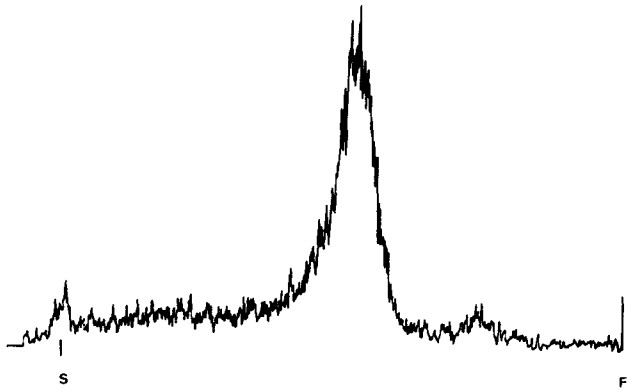


Fig. 3. Radio thin-layer chromatogram of 1-desoxy-D,L-sphinganine 1-phosphonate. Solvent system: n-butanol/acetic acid/water 60 : 20 : 20.

mass spectroscopy of TMS derivative, which was compared with that of sphinganine 1-phosphate (fig. 6).

The tetrakis trimethylsilyl 1-phosphonate and -1-phosphate of sphinganine are characterized by an almost identical fragmentation scheme. The phosphonate gave the following main fragments: *m/e* 638 (M^+-15); *m/e* 566 ($M^+-15-72(CH_2{=}Si(CH_3)_2)$);

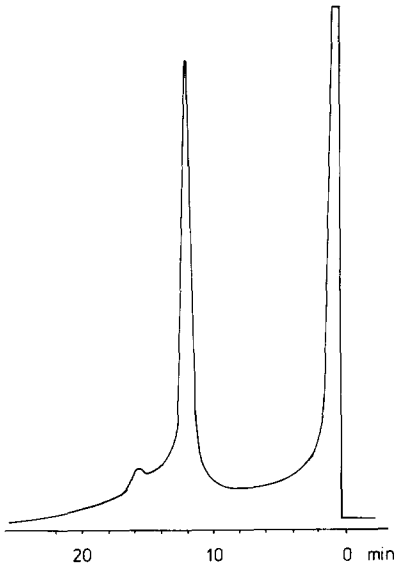


Fig. 4. Radio gas liquid chromatography of tetrakis trimethylsilyl 1-desoxy-D,L-sphinganine 1-phosphonate. 1% SE; 120 cm column, 250°C column temperature; 30 ml Ar/min.

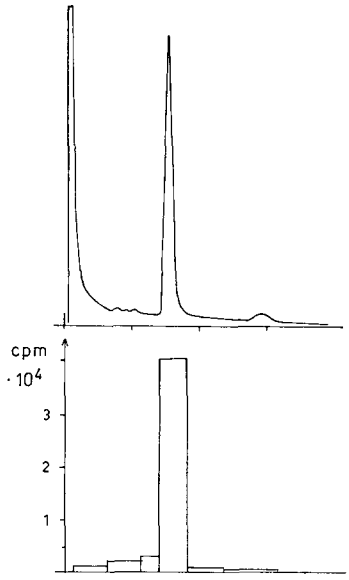


Fig. 5a.

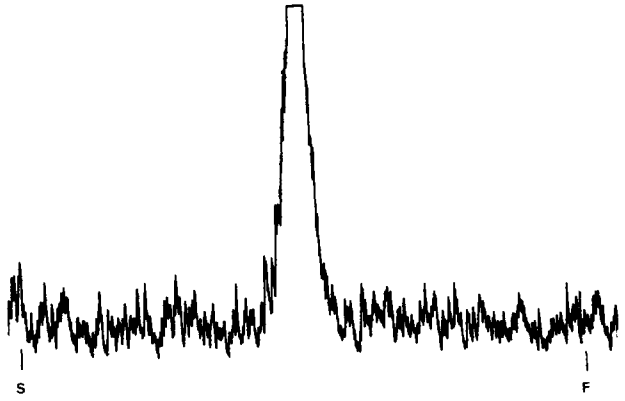


Fig. 5(a) Radio gas liquid chromatogram and (b) radio thin layer chromatogram of long chain aldehyde [1-^{3}H] hexadecanal released from 1-desoxysphinganine 1-phosphonate by periodate oxidation. (a) 15% EGS; 120 cm column; 155°C column temperature; 30 ml Ar/min; (b) solvent system: dichloroethane.

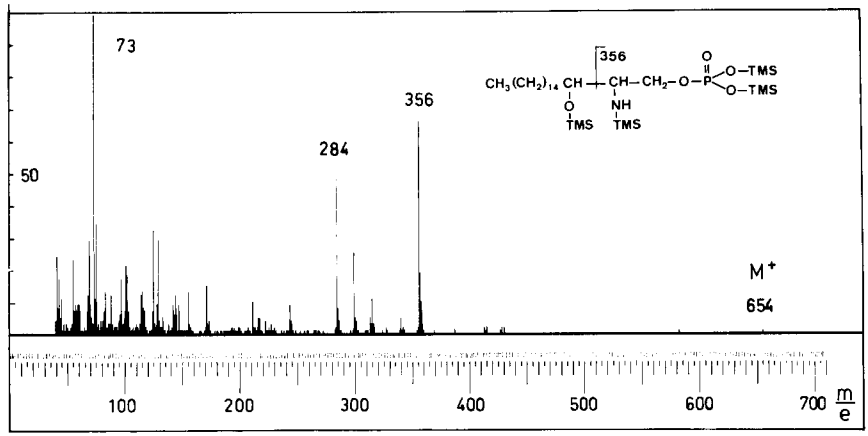


Fig. 6a.

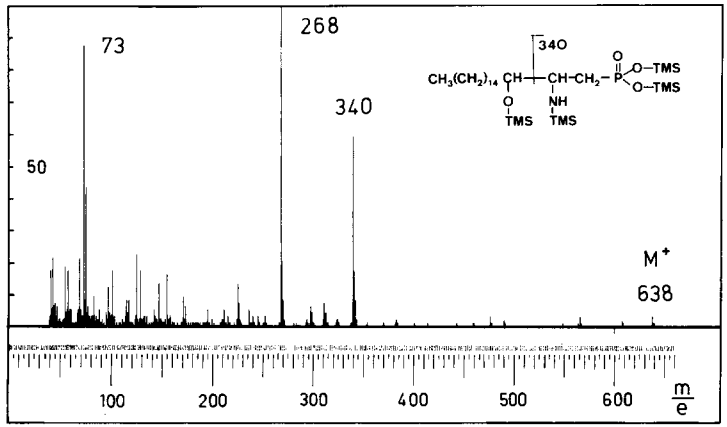


Fig. 6. Mass spectra of (a) tetrakis trimethylsilyl sphinganine 1-phosphate and (b) tetrakis trimethylsilyl 1-desoxy-sphinganine 1-phosphonate.

m/e 340 TMSNH–CH–CH_2–PO $(OTMS)_2^+$; *m/e* 268: TMSNH–CH–CH_2–PO $(OTMS)_2^+$–72; the phosphate ester gave the same fragments enhanced by 16 daltons at 654, 582, 356 and 284. Finally IR-spectroscopy revealed the expected absorption bands, (fig. 7a, b).

The sharp absorption band at 3420 cm^{-1} of the $-NH_3^+$ group in the IR-spectra of the zwitterionic phosphono sphinganine refers to intramolecular hydrogen bonding. It disappears in the IR-spectrum of the dianion with a simultaneous increase of the absorption intensity of the NH_2 group between 3000 and 3700 cm^{-1}. The

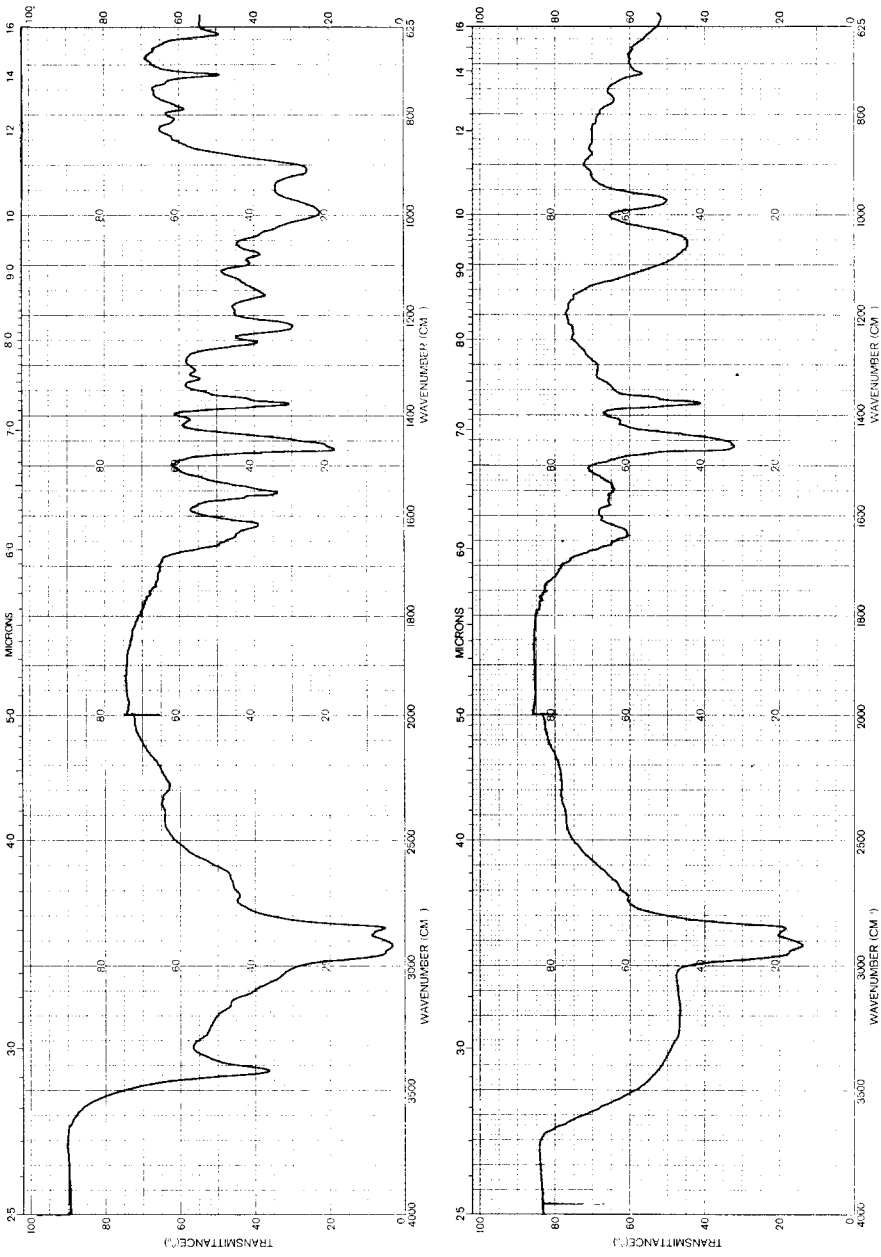


Fig. 7. IR-spectra of (a) 1-desoxy-sphinganine 1-phosphonic acid and (b) disodium salt of 1-desoxysphinganine 1-phosphonate.

loss of the protons leads to the disappearance of the PO–H-absorption between 2550 and 2720 cm^{-1}. The sharp bands at 1550 and 1620 cm^{-1} are contributed by $-NH_3^+$ deformation, which again is strongly reduced in the spectrum of the dianion, whereas the 1640 cm^{-1} band of the NH_2 deformation appears.

The P–O stretching vibration at 1220 cm^{-1} and deformation vibration of $P-O^-$ and P–OH are visible at 1000 and 900 cm^{-1} as strong absorption bands. The P=O absorption disappears in the IR-spectrum of the dianion but that of $P-O^-$ increases at 1060 cm^{-1}.

B. Biochemical studies

1. Studies in vivo

1-Desoxysphinganine 1-phosphonate is a highly toxic compound. When the disodium salt is administered in amounts between 2 and 10 μmoles intravenously to rats, the animals die within a minute. The mode of solubilization is without influence. Even when incorporated in soya lecithin vesicles the toxicity remains unaltered. It could be demonstrated with human blood, that hemolysis occurs even at concentrations of 2×10^{-5} molar. On the other hand the 1-phosphate leaves the erythrocytes intact even up to a 10 times higher concentration; also the growth of *Tetrahymena pyriformis* was arrested when the medium was supplemented with 10 μmoles/l 1-desoxy sphinganine 1-phosphonate.

2. Studies in vitro

Among the rat liver cell fractions which were tested for aldolase activity (mitochondria, 100,000 × *g* supernatent and 100,000 *g* sediment (microsomes)) only the latter fraction proved to be active. The activity could be easily determined by measuring the radioactivity in [1-^{3}H] hexadecanal isolated from the lipid extract by silicic acid chromatography (dichlorethane eluate) and thin layer chromatography (solvent system: 1,2-dichlorethane). Fig. 8 represents the relationship between protein concentrations and product formation and fig. 9 the kinetics of the aldolase reaction with 1-desoxysphinganine 1-phosphonate as substrate. The close structural relationship of sphinganine 1-phosphate and 1-desoxysphinganine 1-phosphonate led us to a comparative study of the aldolase action on these two substrates and a possible inhibitory action of the phosphonate. Michaelis–Menten constants of the 1-phosphonate and of the 1-phosphate were found to be closely identical 1.60×10^{-5} mole/l^{-1}. However the cleavage of 1-desoxy sphinganine 1-phosphonate occurred at a 10-fold slower rate as the sphinganine 1-phosphate (fig. 10). 1-Desoxysphinganine 1-phosphonate is a competitive inhibitor of sphingosine 1-phosphate lyase (aldolase). This is documented in figs. 11 and 12. Fig. 12 represents a Dixon plot [32] from which K_i was determined as 5×10^{-6} mole/l^{-1}.

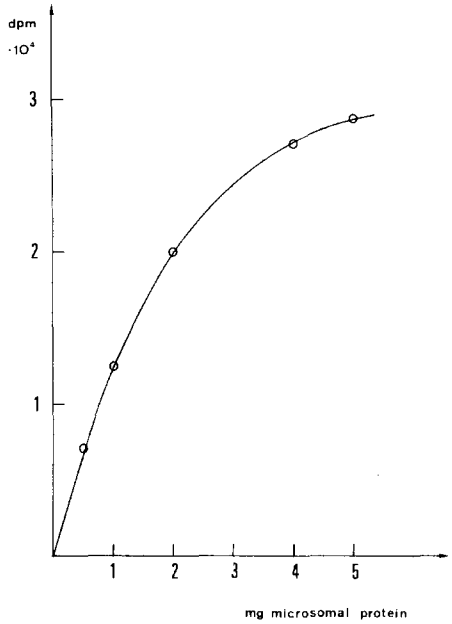


Fig. 8. Dependence of product formation and protein concentration in the aldolase reaction. The reaction mixture contained in 2 ml 0.1 m phosphate buffer pH 7.4 0.2 μmole [3-^{3}H] 1-desoxysphinganine 1-phosphonate, 2 μmol mercaptoethanol, 0.1 μmole pyridoxalphosphate, 2 hr at 37°C.

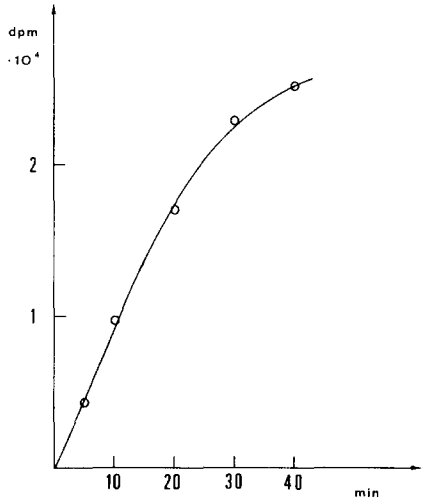


Fig. 9. Reaction kinetics of aldolase with 1-desoxysphinganine 1-phosphonate as substrate. Reaction mixture is given in legend to fig. 8.

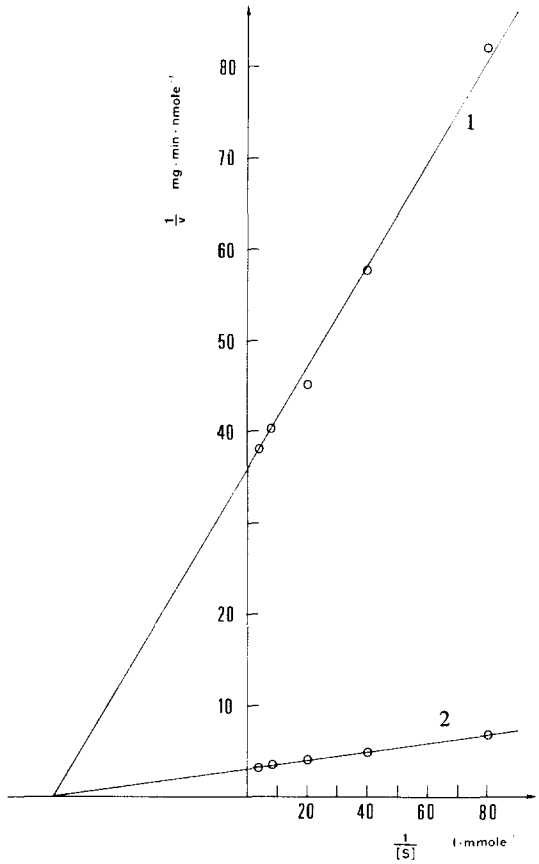


Fig. 10. Comparative Lineweaver–Burk plot of aldolase reactions with (1) 1-desoxysphinganine 1-phosphonate and (2) sphinganine 1-phosphate as substrates.

IV. Discussion

A number of phosphonolipids has been discovered in recent years widely distributed in nature [33–37]. Aminoethylphosphono-analogues of phosphatidylethanolamine, which are the predominant representatives, have also been synthesized [37, 38]. A biosynthetic route of the C–P bond formation of aminoethylphosphonate via an intramolecular rearrangement, reductive amination and decarboxylation of phosphoenol pyruvate has been proposed [39]. Organisms with a high phosphonolipid content such as *Tetrahymena pyriformis* [40, 41] and *Zoanthus sociatus* [41] also contain considerable amounts of 2-amino-3-phosphonopropionic acid and its decarboxylation product 1-aminoethylphosphonic acid, which probably result from

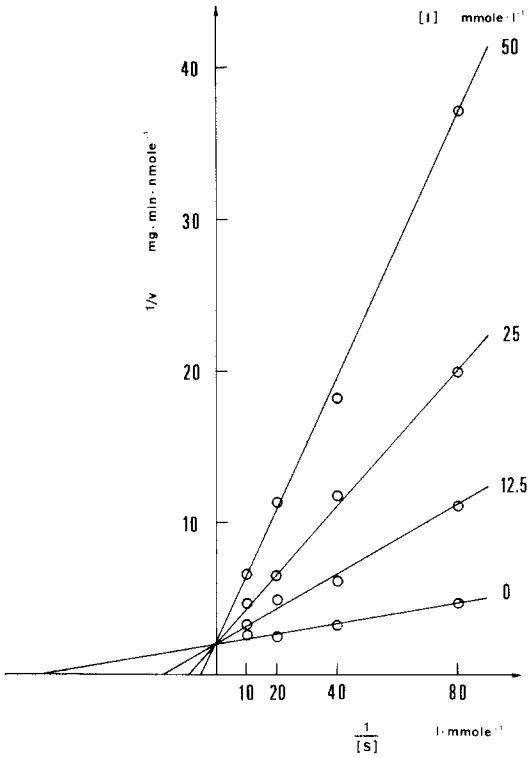


Fig. 11. Competitive inhibition of sphingosine 1-phosphate lyase (aldolase) by 1-desoxysphinganine 1-phosphonate. Conditions of incubations are described in sect. II except the inhibitor concentrations indicated in the figure.

the enzymic degradation of the corresponding phospholipids. Their C–P bond is resistant against hydrolysis. We have synthesized 1-desoxysphinganine 1-phosphonate This compound possesses the negative charges of the phosphate and yet is different from the 1-phosphate ester because of its C–P bond which is resistent to the phosphohydrolase, an enzyme which interferes with the aldolase reaction and secondly imposes a different molecular dimension on the head group. If the phosphonate would fulfill the steric requirements at the active site, it would represent an ideal substrate for studies of the aldolase reaction. We know from previous studies in this laboratory that steric requirements of the aldolase are highly specific towards the 2S, 3R configuration, however the importance of the charged phosphoric acid ester group is unknown. If the function of the phosphate group is the binding of the substrate to a positively charged group at the enzyme surface and thereby supporting the correct orientation of the two optically active centers at C 2 and C 3 changes in the distance of the phosphate head group of the long-chain base should

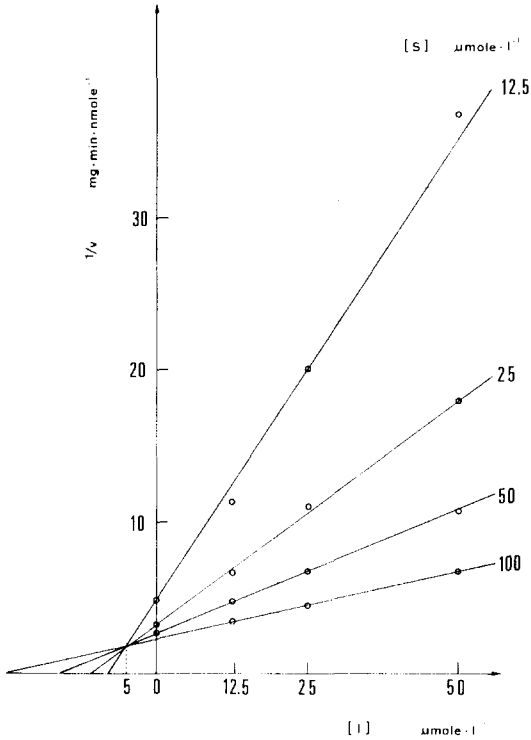


Fig. 12. Dixon plot for the determination of K_i of 1-desoxysphinganine 1-phosphonate. The reaction mixtures contained in a total volume of 2 ml 0.05 molar Tris buffer pH 7.4 : [3-^{3}H] D(+) erythro sphinganine 1-phosphate (0.025, 0.05, 0.1 and 0.2 µmole substrate) 1-desoxy sphinganine 1-phosphonate (0.025, 0.05 and 0.1 µmole) as inhibitor, 40 µmole NaF, 0.1 µmole pyridoxalphosphate, 2 µmole mercaptoethanol and 5 mg microsomal protein. Incubation for 15 min at 37°C.

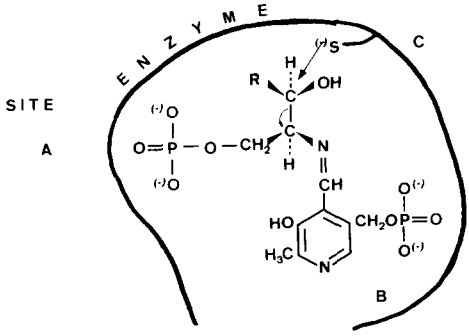


Fig. 13. Proposed mechanism of the sphingosine 1-phosphate lyase (aldolase reaction).

lead to strongly altered binding by electrostatic forces. The substitution of the $-CH_2-O-PO_3^{2-}$ by $-CH_2-PO_3^{2-}$ reduces the length of the molecule by 1.32 Å which is the dimension of the covalently bonded oxygen. The result is a reduced velocity of the phosphonate and a competitive inhibition of the sphingosine-1-phosphate lyase reaction.

[3-^{3}H] 1-desoxysphinganine 1-phosphonate cleaved to the well known products [1-^{3}H] palmitaldehyde and aminoethylphosphonate, although at a recuced rate. On the basis of these and earlier results about the properties of the aldolase (essential SH-groups, pyridoxalphosphate, broad pH-optimum between 6 and 8.5) we would like to propose the following mechanism operative in the lyase reaction (fig. 13). The phosphoryl derivative of the long-chain base has the correct distance to bind to site A. This on the other hand brings the Schiff base of the 2S-amino group and the 3R–OH group in the correct position to site B and the sulfhydryl group at site C for its nucleophilic attack at carbon atom 3. We have little information about the molecular basis of the toxic effect of 1-desoxysphinganine 1-phosphonate. The strong hemolytic effect as compared to the 1-phosphate indicates however a strong disturbance of the membrane structure.

Acknowledgment

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