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Abstract

During recent years signals leading to the early stages of nodulation of legumes by rhizobia have been identified. Plant flavonoids induce rhizobial *nod* genes that are essential for nodulation. Most of the *nod* gene products are involved in the biosynthesis of lipo-oligosaccharide molecules. The common *nodABC* genes are minimally required for the synthesis of all lipo-oligosaccharides. Host-specific *nod* gene products in a given *Rhizobium* species are responsible for synthesis or addition of various moieties to those basic lipo-oligosaccharide molecules. For example, in *R. leguminosarum*, the *nodFEL* operon is involved in the production of lipo-oligosaccharide signals that mediate host specificity. A *nodFE*-determined highly unsaturated fatty acid (*trans*-2, *trans*-4, *trans*-6, *cis*-11-octadecatetraenoic acid) is essential for inducing nodule meristems and pre-infection thread structures on the host plant *Vicia sativa*. Lipo-oligosaccharides also trigger autoregulation of nodulation in pea and, if applied in excessive amounts to a legume, can prevent nodulation and thereby might play a role in competition. During our studies on the biosynthesis of lipo-oligosaccharides, we discovered that, besides the lipo-oligosaccharides, other metabolites are synthesized *de novo* after induction of the *nod* genes. These novel metabolites appeared to be phospholipids, containing either one of the three fatty acids which are made by the action of NodFE in *R. leguminosarum*.

Introduction

Soil bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, collectively called rhizobia, invade the roots of their legume hosts and trigger the formation of a new organ, the nodule. In these root nodules, a differentiated form of the rhizobia, the bacteroid, is able to fix nitrogen into ammonium, which then can be utilized by the plant. The symbiosis occurs in a host-specific way, leading to the definition of cross-inoculation groups in which the bacterial species are classified according their ability to form nitrogen-fixing nodules on host plants. Examples of such cross-inoculation groups are *R. leguminosarum* biovar *viciae* with peas and vetch as hosts, *R. leguminosarum* bv. *trifolii* with clovers as host, *R. leguminosarum* bv. *phaseoli* with bean as host, and *R. meliloti* with alfalfa and sweet clovers as hosts. Several distinct steps of signal exchange between plant and bacterium have been recognized so far as being involved

in the determination of host-specific nodulation. In the first step, plant substances (flavonoids and betaines) excreted from the legume roots induce the transcription of the bacterial nodulation (*nod* or *nol*) genes (Göttfert, 1993; Phillips et al., 1993). The bacterial NodD protein, a transcriptional regulatory protein that presumably interacts with the flavonoids/betaines, contributes to the host specificity of this first step. The molecular chaperone protein GroEL binds directly to NodD and is required for NodD-DNA binding (Long et al., 1994) in order to induce the *nod* or *nol* genes. In the second step, the bacterium, by the means of the activated *nod* or *nol* genes, produces metabolites (Nod metabolites) some of which can act as signals on respective host plants. In all instances characterized so far, these signals are lipo-oligosaccharides (Dénarié et al., 1992; Verma, 1992). Lipo-oligosaccharides are decorated with specific functional groups characteristic for the *Rhizobium* species involved in their synthesis (Fig. 1 and Table 1). Some of the *Rhizobium*-specific decorations have been shown to be essential for proper signaling of the lipo-oligosaccharides to the host plant, thereby initiating the early nodulation events. In this review we try to highlight some novel aspects on the role of lipo-oligosaccharides in root nodule formation.

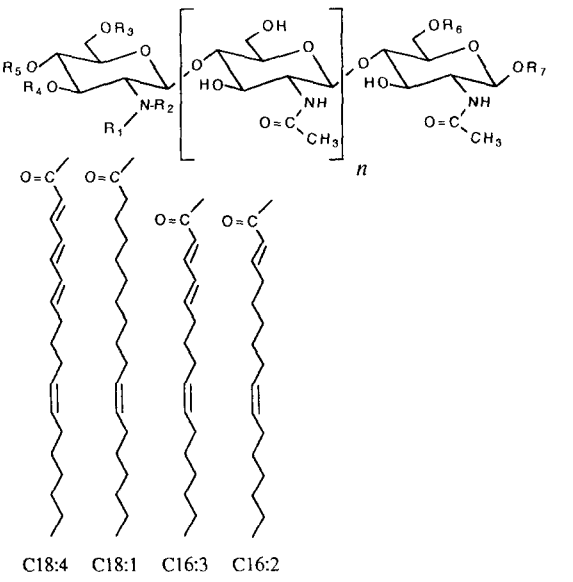

Fig. 1. Basic structure of the rhizobial lipo-oligosaccharides. The most common C18:1 (*cis*-vaccenic acid) and the *nodFE*-derived fatty acyl residues are indicated. The nature of the substituents indicated by R1 to R7 is given in Table 1.

Structures of lipo-oligosaccharides

Since the initial discovery of lipo-oligosaccharides produced by a *R. meliloti* strain (Lerouge et al., 1990), the structures of lipo-oligosaccharides from a sufficient number of rhizobia have been reported to obtain a generalized picture (Bec-Ferté et al., 1993; Carlson et al., 1993; Martínez et al., 1993; Mergaert et al., 1993; Price et al., 1992; Sanjuan et al., 1992; Schultze et al., 1992; Spaink et al., 1991) (Fig. 1 and Table 1). The oligosaccharide backbone of β-1,4-linked *N*-acetyl-D-glucosamines varies in length between three and five sugar units. To the amino nitrogen of the non-reducing end sugar moiety, always a fatty acyl group, the structure of which can vary, is attached. In all rhizobia studied so far lipo-oligosaccharides which are substituted with *cis*-vaccenic acid are found. Such a substituent is not unusual because in rhizobia *cis*-vaccenic acid is the most abundant fatty acid. This is reflected in the fatty acid composition of the major fatty acid-containing pool, the phospholipids. Minor amounts of lipo-oligosaccharides from *R. meliloti* are substituted with ω -OH fatty acids (C16:0 to C26:0) (Demont et al., 1993). It is so far not understood why these presumptive biosynthetic precursors of the ω -OH C28:0 fatty acid, which is part of the lipopolysaccharides of rhizobia, are found in lipo-oligosaccharides of *R. meliloti*.

In all cases studied so far, rhizobial strains harbouring the genes *nodFE(G)* produce additional lipo-oligosaccharides substituted with specific multi-unsaturated fatty acids (Lerouge et al., 1990; Schultze et al., 1992; Spaink et al., 1991). The multiple unsaturation of the acyl residues is thought to be needed for the full biological activity of these specific lipo-oligosaccharide molecules on their respective hosts. In *R. leguminosarum nodFE* are essential for the production of lipo-oligosaccharides that cause mitogenic reactions on the host plant *V. sativa* (Spaink et al.,

Table 1. Lipo-oligosaccharide structures produced by various rhizobia

Strains	R_1	R_2	R_3	R_4/R_5	R_6	R_7	$n=$	Reference
R. leguminosarum bv *viciae*								
wild type	C18:4/C18:1	H	O-acetyl	H	H	H	2,3	Spaink et al.,1991
nodL$^-$	C18:4/C18:1	H	H	H	H	H	2,3	Spaink et al., 1991
nodE$^-$	C18:1	H	O-acetyl	H	H	H	2,3	Spaink et al.,1991
R. meliloti 2011 (pGMI149)								
wild type	C16:2	H	H/O-acetyl	H	SO_3H	H	2,3	Lerouge et al.,1990
nodPQ$^-$	C16:2	H	H/O-acetyl	H	H/SO_3H	H	2,3	
nodH$^-$	C16:2	H	H/O-acetyl	H	H	H	2,3	
AK41	C16:3	H	H/O-acetyl	H	SO_3H	H	1–3	Schultze et al., 1992
R. tropici CFN299	C18:1	CH_3	H	H	H/SO_3H	H	3	Martínez et al., 1993
Rhizobium NGR234	C16:0/C18:1	CH_3	H/carb	H/carb	2-0-CH_3-Fuc/ 2-0-CH_3-3-0-acetyl-Fuc/ 2-0-CH_3-4-0-sulfatyl-Fuc	H	3	Price et al., 1992
R. fredii USDA257	C18:1	H	H	H	Fuc/ 2-0-CH_3-Fuc	H	1–3	Bec-Ferté et al., 1993
B. japonicum								
Type I								
USDA 110	C18:1	H	H	H	2-0-CH_3-Fuc	H	3	Sanjuan et al., 1992
USDA 135	C16:0/C16:1/C18:1	H	H/O-acetyl	H	2-0-CH_3-Fuc	H	3	Carlson et al., 1993
Type II								
USDA 61	C18:1	H/CH_3	H/O-acetyl	H/carb	2-0-CH_3-Fuc/ Fuc	glycerol	2,3	Carlson et al., 1993
A. caulinodans ORS571	C18:0/C18:1	CH_3	H/carb	H	H/arabinosyl	H	2,3	Mergaert et al., 1993

carb: carbamoyl; Fuc: fucosyl

1991; Van Brussel et al., 1992). Common to all *nodFE*-derived fatty acyl residues found in lipo-oligosaccharides so far is an alpha-beta unsaturation in conjugation to the carbonyl group. From a chemical point of view such a configuration means that the delocalization of the π-electrons from the conjugated C=C double bond reduces the positive charge of the carbonyl-C, thereby reducing the possibility of a nucleophilic attack by hydrolytic enzymes. One therefore can predict that the amide bond of an alpha-beta unsaturated fatty acid is much more stable than that of a saturated one. The amide bond of *nodFE*-derived lipo-oligosaccharides therefore should show some protection against possible hydrolytic degradation by the plant. Consistent with this predicted stability is the fact that all those rhizobia containing *nodFE(G)* and therefore producing lipo-oligosaccharides with alpha-beta unsaturated fatty acids (*R. meliloti* and *R. leguminosarum* bv. *viciae*) induce nodule primordia at a distance from the root surface in the uninfected inner layers of the root cortex leading to the formation of indeterminate nodules. In contrast, rhizobia producing only lipo-oligosaccharides without alpha-beta unsaturated fatty acids induce cortical cell divisions just beneath the epidermis giving rise to determinate nodules. The presence of other substitutions depends on the rhizobial strain and are shown in Table 1.

Biological activities of lipo-oligosaccharides

External application of lipo-oligosaccharides, in concentrations varying between 10^{-8} and 10^{-12} *M*, can elicit various effects on root hairs and on cells of the outer and inner cortex of the host plant roots. All lipo-oligosaccharides tested so far cause root hair deformation (HAD) and thick and short roots (TSR) on *Vicia*, but the biological meaning of these effects is not clear (Spaink et al., 1991). Purified lipo-oligosaccharides carrying the respective host-specific decorations of *R. meliloti* or *R. leguminosarum* bv. *viciae* act as mitogens and are able to induce nodule primordia in the inner cortex of the roots of *Medicago sativa* (Truchet et al., 1991) and *Vicia sativa* (Spaink et al., 1991), respectively. These primordia are indistinguishable from the nodule primordia in the first stage of normal nodule organogenesis. In the case of *Medicago* the nodule primordia can even develop into full-grown nodules, albeit free of bacteria (Truchet et al., 1991). In the outer cortex of *Vicia*, mitogenic lipo-oligosaccharides induce the formation of cytoplasmic bridges which are radially aligned, a phenomenon named pre-infection thread (PIT) structure (Van Brussel et al., 1992). Also, the formation of root hair-like structures is stimulated by mitogenic lipo-oligosaccharides (Van Brussel et al., 1992).

Another effect of mitogenic lipo-oligosaccharides, observable only when roots are not shielded from light, is the induction of flavonoid synthesis. The induction of new flavonoids can be monitored by an increase in *nod* gene-inducing activity (INI) of root exudates (Van Brussel et al., 1990). If lipo-oligosaccharides are applied earlier than *Rhizobium* or are applied in large amounts together with *Rhizobium* to the host plant, they can block nodulation (JAN = jamming of nodulation). This interesting finding indicates that lipo-oligosaccharides may play a role in competition (Van Brussel et al., 1993). Like *Rhizobium* bacteria mitogenic lipo-oligosaccharides inactivate a root nodulation factor in pea and it is speculated that this might be the mechanism involved in autoregulation of nodulation (Smit et al., 1993).

Mitogenic lipo-oligosaccharides also induce the early nodulin genes ENOD5 and ENOD12 which are related to the infection process in cells of the root epidermis (Nap and Bisseling, 1990). In the inner cortex ENOD40 is expressed preferentially opposite the proto-xylem poles and ENOD12 only in the cells of the primordium after induction with mitogenic lipo-oligosaccharide. The spatial pattern of ENOD12 and ENOD40 expression, induced by mitogenic lipo-oligosaccharides, corresponds to the pattern after *Rhizobium* infection (see references in Vijn et al., 1993).

Biosynthesis of lipo-oligosaccharides

Proteins encoded by the *nod* genes play an essential role in the biosynthesis of the lipo-oligosaccharides. The NodA, NodB, and NodC proteins are called common Nod proteins because they are functionally interchangeable. They are present in all rhizobia and they are sufficient for the production of a basic lipo-oligosaccharide molecule (Spaink et al., 1991). NodC is homologous to chitin synthases (see references in Spaink et al., 1993b) and it is involved in chitooligomer synthesis from UDP-*N*-acetylglucosamine (Spaink et al., 1993a). Therefore, NodC is thought to assemble the sugar backbone of the lipo-oligosaccharide. Interestingly, the protein most similar to rhizobial NodCs found so far is the FbfA protein which is needed for fruiting body formation by the myxobacterium *Stigmatella aurantiaca* (Schairer, pers. commun.). This is an indication that chitooligomer-derived signal molecules might be involved in morphogenetic processes of bacterial systems as well. In vitro experiments performed with purified NodB show that it can remove the *N*-acetyl group from the non-reducing terminus of chitooligosaccharides (John et al., 1993). In a final step, a fatty acyl residue must be attached to the free amine of the *N*-deacetylated chito-oligosaccharide to obtain a complete lipo-oligosaccharide molecule. The NodA protein may be involved in this acylation step (Spaink et al., 1993a).

Other host-specific Nod proteins are involved in the synthesis or addition of various structural modifications as indicated in Table 1. In *R. meliloti*, the NodPQ proteins function as ATP sulfurylase and APS kinase, respectively, leading to the production of the activated sulfate donor PAPS (Schwedock and Long, 1992). The NodH protein is involved in transferring the sulfate moiety from PAPS to the 6- position of the reducing end of a lipo-oligosaccharide acceptor (Atkinson et al., 1992; Dénarié and Roche, 1992). The NodL protein, which is produced by a number of rhizobial species, is an acetyltransferase involved in the addition of an *O*-acetyl residue at the non-reducing end sugar (Spaink et al., 1993a). In *Rhizobium leguminosarum* bv. *viciae* the NodF and NodE proteins are involved in the production of a lipo-oligosaccharide which carries a highly unsaturated fatty acyl moiety. NodF presumably functions as an acyl carrier protein (Geiger et al., 1991; Shearman et al., 1986) NodE is homologous to β-ketoacyl synthases (Shearman et al., 1986). β-ketoacyl synthase and acyl carrier protein are known to function together in the condensing reaction step of fatty acid biosynthesis.

Are lipo-oligosaccharides the only nod gene-related signals?

Besides the production and secretion of lipo-oligosaccharide signal molecules at least some of the rhizobial Nod proteins have other functions or other functions in addition. The NodO protein is secreted by *R. leguminosarum* bv. *viciae* and is able to form pores in artificial lipid bilayers (Sutton et al., 1993). In *R. trifolii*, a *nodABCIJT*-dependent diglycosyl diacylglyceride is formed which can induce cortical cell division in roots of white clover (Orgambide et al., 1993). Also, some Nod proteins involved in the biosynthesis of lipo-oligosaccharides are involved in the synthesis of intermediates which might represent other potential signals. The presence of a NodC protein is sufficient to allow the synthesis of chitooligomers (Spaink et al., 1993a). Chitooligomers are known to function as signal molecules in their own right on plants during the attack by some phytopathogenic fungi (Ren and West, 1992). The proteins NodFE in a *R. leguminosarum* background are sufficient to allow the synthesis of the *trans*-2, *trans*-4, *trans*-6, *cis*-11-octadecatetraenoic acid. This *nodFE*-derived fatty acid is incorporated into the membrane phospholipids as detailed below (Geiger et al., 1993, 1994).

NodFE-dependent phospholipids

We have shown, that after the induction of the *nodFE* genes, even in the absence of *nodABC*

genes, the *trans*-2, *trans*-4, *trans*-6, *cis*-11-octadecatetraenoic acid, which has an absorbance maximum of 303 nm, is still synthesized, suggesting that the biosynthesis of the unusual fatty acid is completed before it is linked to the sugar backbone of the lipo-oligosaccharide. We also found that the unusual C18:4 fatty acid is linked to the *sn*-2 position of the phospholipids (Geiger et al., 1994). In addition, the phospholipids contain other *nodFE*-derived fatty acids, a *trans*-4, *trans*-6, *cis*-11-octadecatrienoic acid (C18:3) which has an absorption maximum at 225 nm, and a octadecadienoic acid (C18:2) (Geiger et al., 1993). Even when lipo-oligosaccharide signals are produced in a wild type-*Rhizobium* cell, a fraction of all those unusual fatty acids is still bound to all major phospholipids (Geiger et al., 1994). Neither the C18:3 nor the C18:2 fatty acid have been observed so far in lipo-oligosaccharides, suggesting that a still unknown acyl transferase (possibly NodA) responsible for the assembly of the fatty acyl chain to the sugar backbone of the lipo-oligosaccharides, does not transfer all fatty acids synthesized by the action of NodFE to the lipo-oligosaccharides. Rather, it selects for alpha-beta unsaturated fatty acids during transfer. These findings offer interesting possibilities: 1) The phospholipids might be biosynthetic intermediates for the synthesis of lipo-oligosaccharide signals. 2) Phospholipids, containing one of the three different types of *nodFE*-derived fatty acids, might have a signal function on their own. 3) Phospholipids might be a dump for an excess of *nodFE*-derived fatty acids. 4) Phospholipids, containing multi-unsaturated fatty acids might have another, hitherto unexpected function.

Effect of nodFE overexpression on growth rate

Organisms normally adapt to lower temperatures by changing the composition of their membranes (Murata et al., 1992). In various prokaryotic and eukaryotic systems, the relative amount of unsaturated fatty acids in the membranes is increased at lower temperatures. It is thought that the loss of membrane fluidity associated with a decrease

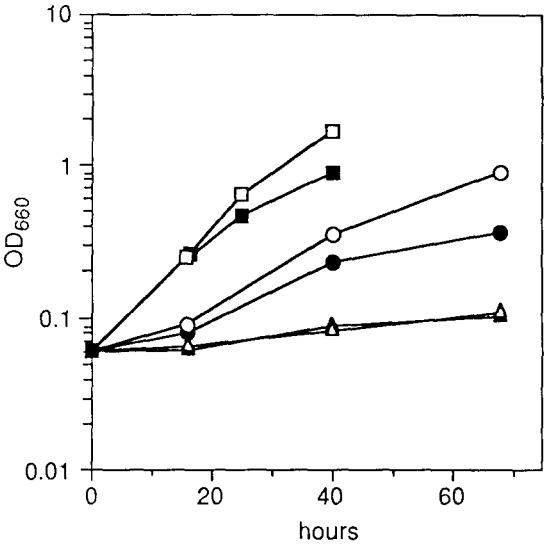

Fig. 2. Growth of *Rhizobium leguminosarum* RBL5560.pMP1255 cultures after flavonoid induction. LPR5045 is a Sym plasmid-cured *Rhizobium* strain. RBL5560 is LPR5045 containing the *R. leguminosarum* bv. *viciae* Sym plasmid pRL1JI *mep*::Tn5 (Zaat et al., 1987). pMP1255 harbours cloned *nodFE* of pRL1JI in an IncQ plasmid (Geiger et al., 1991). Cells of strain RBL5560.pMP1255 were grown in B^- medium in the absence (□ ○ △) or presence (■ ● ▲) of the inducer naringenin (1.5 u*M*) at 20°C (□■), 16°C (○ ●) or at 4°C (△▲).

in temperature might be, at least partially, compensated by a fluidity increase due to the presence of more unsaturated fatty acids. Expression of the genes *nodFE* leads to increased amounts of unsaturated fatty acids found in the phospholipids of *R. leguminosarum* membranes (Geiger et al., 1993; Geiger et al., 1994). We noticed that *R. leguminosarum* cultures, in which *nodFE* are overproduced, show longer generation times at normal (29°C) growth temperature than control cultures which had not been induced (Fig. 2). Induced cultures of RBL5560.pMP190 that contain only one copy of *nodFE* on the Sym plasmid but an empty IncQ plasmid, without *nodFE*, show normal short generation times (data not shown).

The reduced growth of *nodFE*-overproducing *R. leguminosarum* cultures could be explained by an increased membrane fluidity which would be too high for optimal growth rates. If true, one would expect that at reduced temperatures in the NodFE-overexpressed situation, the contents of unsaturated fatty acids in the membranes causes

a higher fluidity allowing better growth than that shown by cultures that do not express NodFE. Such a strategy might lead to the construction of *Rhizobium* strains with increased cold tolerance. We analyzed growth rates of non-induced and *nodFE*-induced cultures at normal and reduced growth temperatures and found that also at lower temperatures (16°C), the growth in the *nodFE*-induced culture was always significantly slower than in the uninduced control cultures (Fig. 2). At 4°C hardly any growth was noticed in non-induced or *nodFE*-induced cultures. We therefore think that the reduced growth rates observed after overproduction of NodFE are not due to a change in membrane fluidity. The most likely explanation is that such high levels of NodFE are interfering with normal fatty acid synthesis of the cell, thereby slowing down growth.

Biological activity of NodFE-dependent phospholipids

We tested NodFE-dependent phospholipids in various bioassays that are normally used to analyze lipo-oligosaccharides. We could not detect any induction of nodule meristems, preinfection threads or INI after incubation of *Vicia sativa* in the presence of NodFE-dependent phospholipids. We also tested the effects of fatty acids obtained after mild alkaline hydrolysis of the phospholipids. Such fatty acids obtained from *nodFE*-independent or *nodFE*-dependent phospholipids had no effect on HAD or TSR, if applied alone. In the presence of rhizobial strains, lacking *nodE*, and which normally make only non-mitogenic lipo-oligosaccharides (i.e. RBL5580.pMP1060) a stimulation of TSR and HAD is observed by addition of fatty acids derived from phospholipids that contain *nodFE*-determined fatty acids but not by monounsaturated or saturated fatty acids (data not shown). We could even show, that *nodFE*-determined fatty acids obtained by hydrolysis of phospholipids in combination with RBL5580.pMP1060 cause INI on *V. sativa* (Table 2). An explanation for these results is, that *nodFE*-determined fatty acids are taken up by rhizobia and are attached to *N*-deacetylated chitooligomers during lipo-oligosaccharide biosynthesis to form mitogenic lipo-oligosaccharides which are able to cause INI. Experiments are under way to see whether lipo-oligosaccharides produced by RBL5580.pMP1060 (*nodE*$^-$) in the presence of

Table 2. Induction of INI by *nodE*-deficient *Rhizobium* in the presence of NodFE-derived fatty acids

Hydrolyzed phospholipids from	Strain RBL5580 pMP1060	INI (units of β-galactosidase) 4 days	7 days
LPR5045 - nar pMP281	-	210	130
pMP1255	+	223	596
LPR5045 + nar pMP281	-	40	63
pMP1255	+	704	2400
LPR5045 + nar pMP281	-	30	36
	+	150	605
LPR5045 + nar pMP1255	-	1	47
	+	150	1559
RBL5580 + nar pMP1060	-	30	67
	+	110	430
-	+	70	681
-	RBL5601	3028	6591
-	-	40	50

INI was measured after 4 and 7 days of co-culture of *V. sativa* and *Rhizobium* (strain RBL5580.pMP1060 or RBL5601)(van Brussel et al., 1990) in the presence of phospholipid hydrolysates and 0.004% CHAPS. LPR5045 is a Sym plasmid-cured *Rhizobium* strain. RBL5601 is LPR5045 containing Sym plasmid pRL1JI. RBL5580 is LPR5045 containing pRL1JI::Tn1831 (deletion of *nodELMNO*). pMP281 (cloned *nodD* of pRL1JI in IncP). pMP1060 (cloned *nodL* of pRL1JI in IncP). pMP1255 (cloned *nodFE* of pRL1JI in IncQ). Hydrolyzed phospholipids used per INI assay were obtained from about 10^{11} of uninduced or naringenin-induced (nar) cells by a Bligh and Dyer extraction prodcedure. $CHCl_3$-soluble material was chromatographed on HPLC silica gel (Geiger et al., 1994) and allowed the separation of phospholipids from other lipids. Phospholipids were subsequently subjected to mild alkaline hydroysis (0.2 *M* NaOH in MeOH, 1 h, 20°C) and fatty acids were obtained in the $CHCl_3$ phase after another Bligh and Dyer partition.

a chemically synthesized *trans*-2, *trans*-4, *trans*-6, *cis*-11-octadecatetraenoic acid (Verduyn et al., 1992) are indeed mitogenic lipo-oligosaccharides and give INI, NOI, and PIT.

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