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# Identification of a species-specific inhibitor of glycosylphosphatidylinositol synthesis

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**Glycosylphosphatidylinositol (GPI)-anchoring represents a mechanism for attaching proteins to the cell surface that is used among all eukaryotes. A common core structure, EthN-P-Man$_3$-GlcN-PI, is synthesized by sequential transfer of sugars and ethanolamine-P to PI and is highly conserved between organisms. We have screened for natural compounds that inhibit GPI-anchoring in yeast and have identified a terpenoid lactone, YW3548, that specifically blocks the addition of the third mannose to the intermediate structure Man$_2$-GlcN-acylPI. Consistent with the block in GPI synthesis, YW3548 prevents the incorporation of [$^3$H]*myo*-inositol into proteins, transport of GPI-anchored proteins to the Golgi and is toxic. The compound inhibits the same step of GPI synthesis in mammalian cells, but has no significant activity in protozoa. These results suggest that despite the conserved core structure, the GPI biosynthetic machinery may be different enough between mammalian and protozoa to represent a target for anti-protozoan chemotherapy.**

*Keywords*: GPI/mannosyltransferase/protozoa/yeast

## Introduction

Many proteins are attached to the cell surface by a GPI-anchor which contains a conserved core structure that is common among all eukaryotes (McConville and Ferguson, 1993). The common structure consists of EthN-P-6Manα-1,2Manα-1,6Manα-1,4GlcNα-1,6*myo*-inositol-P-lipid and is assembled by sequential addition of the sugar components and ethanolamine phosphate to phosphatidylinositol. The sugars as well as the lipid moieties are subjected to species- and stage-specific modifications such as addition of ethanolamine or sugars on mannose residues of the GPI-core structure (McConville and Ferguson, 1993). It has been proposed that GPI-anchor synthesis occurs on the cytoplasmic side of the ER (Vidugiriene and Menon, 1993, 1994; Takeda and Kinoshita, 1995). Once assembled, the complete precursor may be flipped into the ER lumen where it is transferred *en bloc* to the C-terminal carboxyl group of the protein. Anchor addition is thought to be a transamidation reaction in which the C-terminal hydrophobic signal peptide found on proteins to be GPI-anchored is replaced by the GPI-precursor (Maxwell *et al.*, 1995; Riezman and Conzelmann, 1998).

Progress has been made in the characterization of the GPI biosynthetic machinery. The identification of lymphoma cell and yeast mutants lacking the expression of GPI-anchored proteins at the cell surface has allowed the identification of several genes involved in GPI-anchor biosynthesis. In both organisms, mutants of three complementation classes have been isolated that inhibit the addition of GlcNAc to PI (Leidich *et al.*, 1994; Takeda and Kinoshita, 1995 and references therein). In animal cells, two mutants are defective in the deacetylation of GlcNAc-PI to give GlcN-PI (Mohney *et al.*, 1994; Stevens *et al.*, 1996). Mutants in dolichol-*P*-mannose synthase also block GPI-mannosylation, but also affect protein glycosylation (Chapman *et al.*, 1980; Orleans, 1990). In both systems, mutant cells are available that block addition of the third mannose to the GPI core structure (Benghezal *et al.*, 1995; Takahashi *et al.*, 1996), and in animal cells, a mutation affects the transfer of the terminal ethanolamine-P onto the third mannose (Sugiyama *et al.*, 1991). Mutants have also been isolated that block attachment of the complete GPI-precursor to the protein (Hamburger *et al.*, 1995; Benghezal *et al.*, 1996).

Several of the corresponding genes have been cloned by complementation of the GPI defects, including the *PIG-A*, *PIG-H*, *PIG-F*, *PIG-C* and *PIG-B* from mammalian cells and the *GPI1*, *GPI2*, *GPI3/SPT1, GAA1* and *GPI8* genes from yeast (Inoue *et al.*, 1993; Kamitani *et al.*, 1993; Miyata *et al.*, 1993; Hamburger *et al.*, 1995; Leidich *et al.*, 1995; Schönbächler *et al.*, 1995; Benghezal *et al.*, 1996; Inoue *et al.*, 1996; Takahashi *et al.*, 1996). It is interesting to note that at least three gene products are required for the first biosynthetic step in both organisms and that at least two gene products are required to transfer the preformed anchor to the protein. Some of the GPI biosynthetic enzymes are conserved between organisms. The mammalian homologue of the *GPI2* gene from yeast was identified by sequence homology (20% identity) and complements class C lymphoma cell mutants (Inoue *et al.*, 1996). The identification of the *SPT14* gene was also based on sequence identity (44%) with the human *PIG-A* gene (Schönbächler *et al.*, 1995).

Inhibitors have also been used to study the GPI-anchor biosynthetic pathway. 2-Fluoro-2-deoxyglucose and amphomycin are known inhibitors of dolichol-*P*-mannose synthase and they block the mannosylation of GPI-intermediates (Takami *et al.*, 1992). Treatment of mammalian cells or trypanosomes with the amino sugar mannosamine



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blocks GPI-biosynthesis (Lisanti *et al.*, 1991; Ralton *et al.*, 1993). In both organisms, mannosamine appears to block the addition of the third mannose to the intermediate structure (Pan *et al.*, 1992; Ralton *et al.*, 1993; Sevlever and Rosenberry, 1993). The serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was shown to inhibit GPI-anchoring in African trypanosomes (Masterson and Ferguson, 1991). PMSF was shown to specifically block the inositol acylation process that converts glycolipid precursor A into precursor C, which is required for the subsequent addition of ethanolamine phosphate (Güther *et al.*, 1994; Güther and Ferguson, 1995). PMSF is not active in mammalian cells (Güther *et al.*, 1994). Diisopropylfluorophosphate also affects the inositol deacylation step in trypanosomes (Güther and Ferguson, 1995). These inhibitors do not seem to be specific for GPI-anchoring.

Rational drug design appears to be a powerful approach for the identification and development of new drugs (Petsko, 1996). However, this process requires detailed information on biosynthetic pathways as well as knowledge of the precise structure of the target enzyme. In the case of GPI-anchoring, many of the genes required for the process have been identified, but none of the enzymes has been purified, nor is there any three-dimensional structural information available. Screening of natural products remains the most commonly used procedure for the discovery of new compounds (Verdine, 1996). Therefore, we have applied a screening approach to search for natural compound inhibitors of GPI-anchor synthesis in yeast. We have identified a compound that blocks the incorporation of the third mannose onto the GPI-intermediate structure $Man_2$-GlcN-acylPI. This terpenoid lactone blocks GPI-synthesis in yeasts and in mammalian cells, but not in protozoa suggesting that there are significant interspecies differences in GPI-synthesis.

## Results

### *Screen to identify compounds that block Gas1p transport to the Golgi*

In order to identify compounds that inhibit GPI-anchoring in yeast, we have carried out a screen for natural product inhibitors. The screen is based on the maturation of the major yeast GPI-anchored protein Gas1p. Gas1p occurs as a 105 kDa form in the ER and upon transport to the Golgi, its core glycan chains are elongated giving rise to a form of 125 kDa. Transport of Gas1p to the Golgi depends on addition of the GPI-anchor, on ceramide synthesis and on early secretion (*SEC*) genes (Conzelmann *et al.*, 1988; Nuoffer *et al.*, 1993; Horvath *et al.*, 1994; Doering and Schekman, 1996). We have exploited these transport requirements to set up a screen of natural products in which wild type yeast cells are incubated with crude extracts from fungi and bacteria for 90 min. Total protein is extracted, separated by SDS–PAGE and transferred to a nitrocellulose filter which is decorated with polyclonal antibody directed against Gas1p (Figure 1). Once a compound is found to cause the accumulation of Gas1p in its 105 kDa form, the maturation of the non-GPI-anchored protein, carboxypeptidase Y (CPY) is tested by Western blot analysis and the compound is subsequently isolated. This screening procedure has already led to

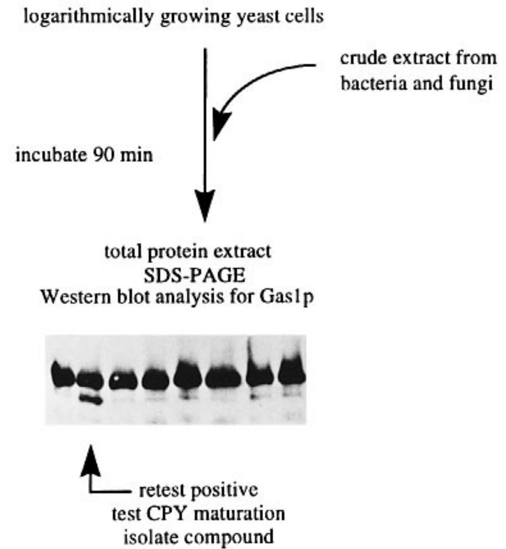


**Fig. 1.** Outline of the applied screening procedure. The lower band in the Western blot is the 105 kDa form of Gas1p indicative of a defect in GPI-anchoring and/or protein transport.

the identification of myriocin, an inhibitor of ceramide synthesis (Horvath *et al.*, 1994). Based on the maturation inhibition of Gas1p, we have shown that ceramide synthesis represents a selective transport requirement for GPI-anchored proteins in yeast.

We have now identified a compound, YW3548, that caused the accumulation of the ER form of Gas1p while the maturation of CPY as tested by Western blot analysis was not affected. *In vivo*, the compound is active at concentrations as low as 0.25 μM and its effect appears to be reversible (data not shown).

### *Identification of YW3548*

YW3548 which is a metabolite from *Codinea simplex*, was chemically purified and each step of purification was followed by the killing activity of the compound on a yeast lawn and by testing Gas1p maturation by Western blot analysis. The structure as determined by IR, MS and NMR, was found to be a terpenoid lactone (see Figure 6A for the structure of YW3548).

### *Gas1p transport is specifically blocked by YW3548*

To confirm the Western blot data, we performed pulse–chase analysis in the absence or presence of YW3548. Wild type cells were preincubated either with YW3548 or methanol as control for 10 min. They were radiolabeled with [$^{35}$S]cysteine/methionine and chased for the indicated times. After cell lysis, Gas1p and CPY were immunoprecipitated and the immune complexes were analyzed by SDS–PAGE, followed by fluorography. YW3548 abolished the maturation of Gas1p from the 105 kDa form to the 125 kDa form (Figure 2A). Under the same conditions the kinetics of CPY transport from the ER (P1) to the Golgi (P2) to the vacuole (P3) were not changed in comparison to the methanol control (Figure 2B). Furthermore, we tested the maturation and secretion of invertase which additionally allowed us to address whether protein glycosylation is affected by YW3548. Invertase

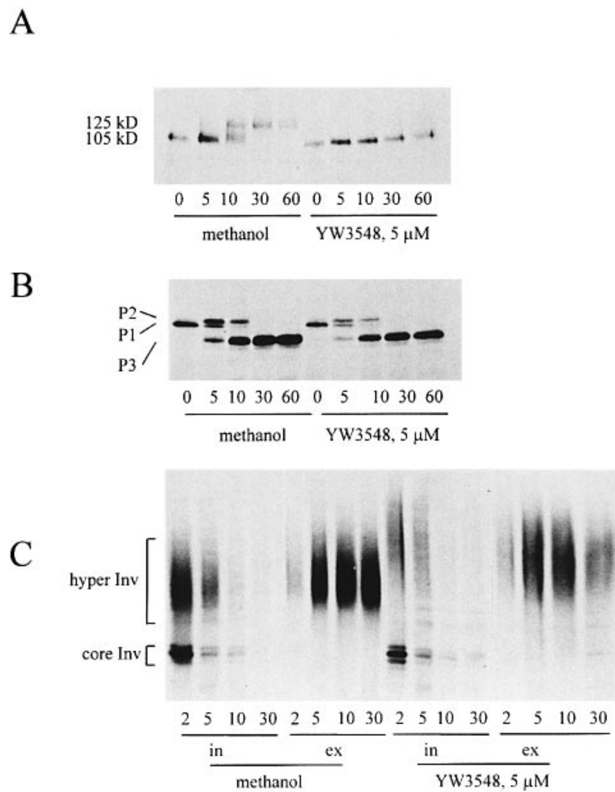


**Fig. 2.** Biogenesis of Gas1p, CPY and invertase. Pulse–chase analysis of three secretory markers was performed in the presence or absence of YW3548, a GPI-anchored protein, Gas1p (**A**), a vacuolar hydrolase, CPY (**B**) and a periplasmic protein, invertase (**C**). Cells were preincubated with YW3548 or with methanol for 10 min before a 5 min pulse. After the chase (time is indicated in min), cells were lysed and Gas1p or CPY was immunoprecipitated using polyclonal antisera. The immune-complexes were separated by SDS–PAGE and analyzed by fluorography. The migration of the immature (105 kDa) and mature (125 kDa) forms of Gas1p, and the ER (p1), Golgi (p2) and mature (P3) forms of CPY are shown. The maturation of the glycoprotein invertase is shown in the presence of YW3548 or methanol. 'core Inv' indicates the ER form and 'hyper Inv' the hyperglycosylated form of the protein. 'in' refers to invertase found in the cells whereas 'ex' refers to invertase secreted into the medium. The chase times are indicated in min.

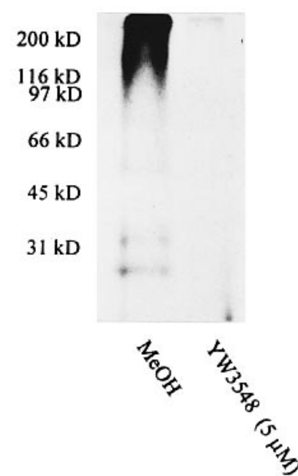


**Fig. 3.** GPI-anchor attachment is inhibited by YW3548. [$^3$H]*myo*-inositol labeling of wild type cells (RH448) after a 10 min preincubation with methanol or YW3548. After a 30 min pulse, total cellular proteins were extracted, separated by SDS–PAGE (10% gel) and analyzed by fluorography.

occurs as a 79–83 kDa ladder in its core-glycosylated ER form. Upon transport to the Golgi, the core glycans are greatly elongated by outer chain mannose residues in α-1,6, α-1,2 and α-1,3 linkages and it appears as a high molecular weight smear of 100–150 kDa. Hyperglycosylated invertase is then secreted into the periplasm. We found that the kinetics of secretion were not changed in the presence of the compound (Figure 2C). We also observed that outer chain addition occurred, but the hyperglycosylated form of invertase ran at a slightly higher apparent molecular weight in the presence of YW3548 as compared with the methanol control. We conclude that YW3548 specifically blocks the transport of Gas1p to the Golgi while the transport and the glycosylation of other secretory marker proteins is not inhibited.

### YW3548 exhibits a general effect on GPI-anchoring

Inhibition of several processes can lead to the Gas1p maturation defect seen upon incubation of yeast cells with YW3548. The compound could specifically affect the transport of GPI-anchored proteins, GPI-anchor biosynthesis or the attachment of the anchor to the protein. In order to differentiate between the first and latter possibilities, we labeled cells with [$^3$H]*myo*-inositol, which can be incorporated into GPI-anchored proteins due to the presence of inositol in the GPI-core structure. Wild type cells were preincubated with YW3548 or methanol for 10 min then labeled with [$^3$H]*myo*-inositol for 30 min. Total protein was extracted, separated by SDS–PAGE and the signal was detected by fluorography. In the absence of the YW3548, most of the radioactivity was incorporated into glycoproteins found as a high molecular weight smear at the top of an SDS gel and into some proteins of lower molecular weight seen as distinct bands (Figure 3). Upon incubation with the compound, however, inositol incorporation into proteins was completely abolished suggesting that YW3548 blocks either synthesis of the GPI-precursor or its transfer to the protein. These data also show that the compound affects biosynthesis of all GPI-anchored proteins.

### YW3548 causes the accumulation of $Man_2$-GlcN-acylPI in vitro and in vivo

To distinguish between a block in precursor synthesis or anchor attachment, we developed an *in vitro* system for GPI-anchor synthesis. This system is based on a crude membrane preparation from yeast which is incubated with [$^{14}$C]GDP-mannose. After 60 min incubation at 30°C, total membrane lipids were extracted and analyzed by TLC. The membranes were found to be competent for the synthesis of the whole range of GPI-intermediates including the complete GPI-precursor (Figure 4, lane 1). Upon incubation of the membranes with YW3548 for 5 min before addition of the label, the synthesis of the complete precursor as well as of less polar intermediate structures was decreased and one intermediate structure was found to accumulate predominantly (lane 2, labeled $Man_2$). This same intermediate was found to accumulate when membranes were labeled with [$^3$H]UDP-GlcNAc (data not shown).

To verify that this *in vitro* result reflects the *in vivo* action of the inhibitor, we tested whether YW3548 caused

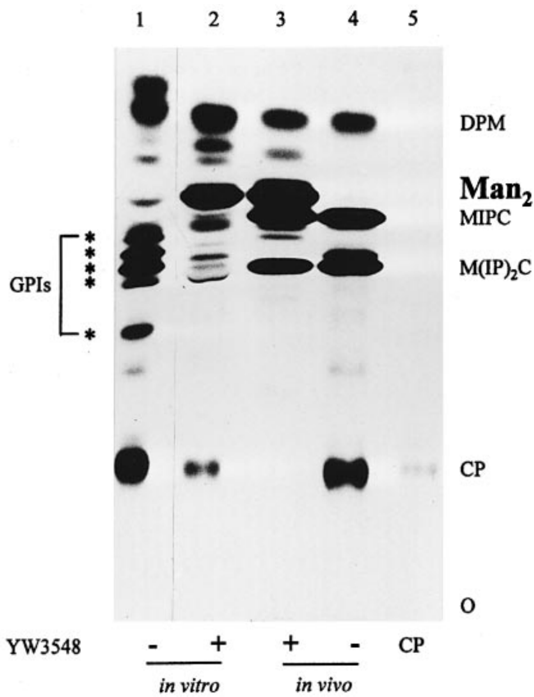


**Fig. 4.** Effect of YW3548 on *in vitro* and *in vivo* GPI labeling experiments. Total yeast membranes were incubated with [$^{14}$C]GDP-Man for 60 min in the presence of 170 nM YW3548 (+) or methanol (–) followed by lipid extraction and analysis by TLC (*in vitro*). GPI intermediates are marked by (*), CP indicates the position of the complete precursor which was also loaded as a standard. Labeling of *pmi40* cells with [$^3$H]Man after a 20 min preincubation with YW3548 or with methanol. Lipids were analyzed by TLC after a 30 min pulse (*in vivo*). The accumulated intermediate is referred to as $Man_2$. DPM, dolichol-*P*-mannose; MIPC, mannosylinositolphosphoceramide; $M(IP)_2C$, mannosyldiinositolphosphoceramide; O, origin.

the accumulation of an intermediate structure in intact cells. To enhance incorporation of radioactive mannose into lipids, a mutant strain with a temperature sensitive allele of phosphomannose isomerase, *pmi40*, was used. *pmi40* cells incorporate mannose into glycolipids and glycoproteins with high efficiency when shifted to the non-permissive temperature (Sipos *et al.*, 1994). *pmi40* cells were labeled with [$^3$H]mannose for 30 min after a 20 min shift to non-permissive temperature in the presence of YW3548. Total lipids were extracted and analyzed by TLC and fluorography. Mainly ceramides, as found by comigration with [$^3$H]*myo*-inositol labeled lipids, dol-*P*-man and the complete GPI-precursor were labeled with [$^3$H]mannose in the methanol control (lane 4) (Sipos *et al.*, 1994). In the presence of YW3548, one GPI intermediate accumulated in *pmi40* cells that comigrated with the glycolipid accumulated *in vitro* and the synthesis of the complete precursor was abolished (lane 3). YW3548 did not affect sphingolipid synthesis since the amounts of MIPC and $M(IP)_2C$ remained the same in the presence or absence of the compound (lanes 3 and 4). The accumulation of this intermediate ($Man_2$) was also observed after labeling with [$^3$H]*myo*-inositol, but was only detectable with our TLC systems if lipids were analyzed by 2D-TLC (data not shown). These results suggest that YW3548 affects the same GPI-biosynthetic step *in vitro* and *in vivo*.

The intermediate structures that accumulate in the presence of YW3548 *in vivo* or *in vitro* were scraped off

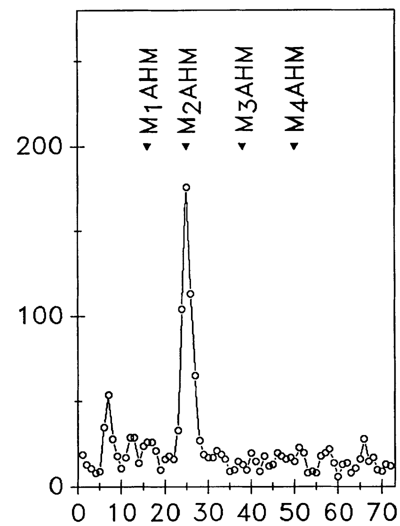


**Fig. 5.** Analysis of the accumulated GPI intermediate. The neutral glycan headgroup of the *in vitro* accumulated intermediate was prepared by dephosphorylation followed by nitrous acid deamination and reduction and its structure was determined by its migration on Dionex HPAEC by comparison with appropriate standards.

the TLC plate and converted into neutral glycans by nitrous acid deamination and borohydride reduction. They were then analyzed by Dionex HPEAC or by comigration with a standard GPI-intermediate ladder on HPTLC plates (Schneider *et al.*, 1993). By both means, a $Man_2$-anhydro-mannitol accumulated showing that the structure of the intermediate was likely to be $Man_2$-GlcN-acylPI (Figure 5). Furthermore, this $Man_2$ intermediate structure was found to be resistant to treatment with PI-PLC suggesting that it was acylated on the inositol ring (data not shown).

### Generation of derivatives of YW3548

To obtain information on the active groups of the molecule, we generated and/or isolated several derivatives of YW3548 (Figure 6B–E) and tested their activity using the *in vitro* system, by Western blot analysis after a 90 min incubation of cells with the compound or by halo assay. We found that the changes which mainly affected the lactone ring of YW3548 or its disposition caused a loss of activity of about 500- to 1000-fold (Table I). In particular, YW3699 is identical to YW3548 except for the lactone ring, but it is 1000-fold less active. Therefore, it seems that the lactone ring is essential for inhibitory activity.

### Effects of YW3548 on other systems

We have shown that the compound YW3548 is a powerful inhibitor of the addition of the third mannose onto the GPI intermediate $Man_2$-GlcN-acylPI in yeast. Since the GPI-core structure from all eukaryotes has been shown to be highly conserved, it was interesting to test if YW3548 is also active in other eukaryotes.

In parasitic protozoa, GPI-anchoring is extensively used to anchor surface coat proteins and the first structural analysis of the GPI-anchor was performed on the VSG surface protein from *Trypanosoma brucei* (Ferguson *et al.*, 1988). We therefore tested for activity of YW3548 and its derivatives in *in vitro* GPI synthesis systems with

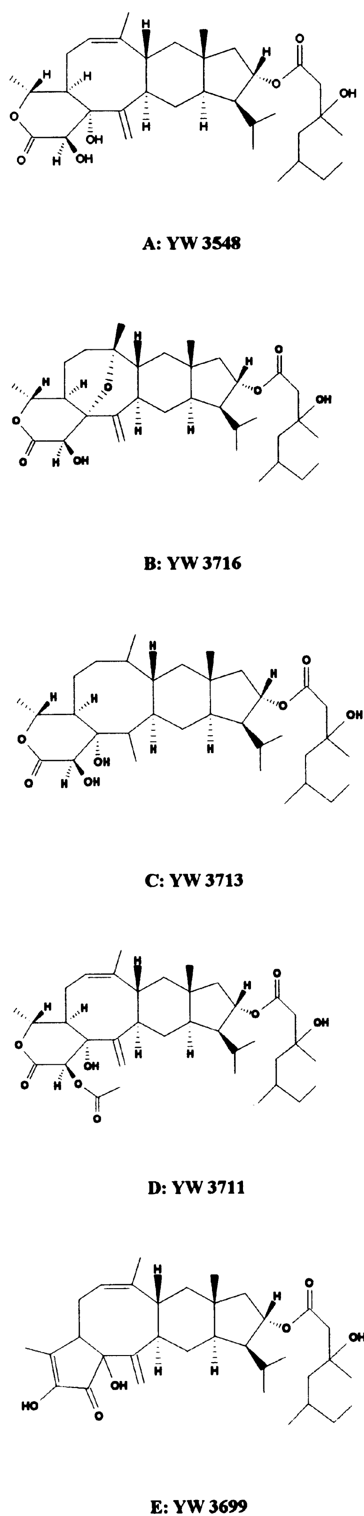


**Fig. 6.** The structure of YW3548 and related compounds. The structures of YW3548 and related compounds were determined by IR, NMR and mass spectroscopy, YW3548 (**A**), YW3716 (**B**), YW3713 (**C**), YW3548, YW3711 (**D**) YW3699 (**E**).

membranes derived from *T.brucei* and *Plasmodium falciparum* (Figure 7). Interestingly, no GPI synthesis defect was observed in the presence of YW3548 or any of its derivatives and no intermediate structure accumulated (Figure 7B and C in comparison with the accumulation of the $Man_2$ intermediate in yeast Figure 7A). There were minor differences in relative peak heights, but these were not reproducible (data not shown). Furthermore, we observed that less radioactivity was incorporated into protozoan GPI-intermediates upon incubation with YW3548. Also, *in vitro* GPI-labeling experiments in *Paramecium primaurelia* and *Toxoplasma gondii* showed that YW3548 does not affect GPI-anchor synthesis (C.Sütterlin, N.Azzouz, C.F.Zinecker, P.Gerold, R.T. Schwarz and H.Riezman, unpublished results) confirming our results obtained with other protozoan species.

**Table I.**

| Compound | Minimal inhibitory concentration[a] |
|---|---|
| YW3548 (Figure 6A) | 3.4 nM |
| YW3716 (Figure 6B) | 17 µM |
| YW3713 (Figure 6C) | 1.7 µM |
| YW3711 (Figure 6D) | 3.2 µM |
| YW3699 (Figure 6E) | 3.5 µM |

[a]As determined by *in vitro* GPI synthesis in yeast.

We also tested whether YW3548 showed inhibitory activity in a medically relevant fungus, *Candida albicans*. Total membranes from a wild type *C.albicans* strain were prepared. These membranes were then incubated with [$^{14}$C]GDP-Man for 60 min in the presence or absence of YW3548. Lipids were extracted and analyzed by TLC. *Candida* membranes prepared in this manner did not synthesize GPIs as efficiently as membranes from *S.cerevisiae* and the complete precursor could not be detected. However, upon incubation of these membranes with YW3548, the accumulation of an intermediate structure comigrating with the yeast $Man_2$-intermediate was observed (Figure 8A, arrow). Furthermore, we found that in *C.albicans*, no [$^{3}$H]*myo*-inositol was incorporated into proteins in the presence of YW3548 (data not shown). YW3548 was found to be equally toxic to *C.albicans* and *S.cerevisiae* as tested by halo assay.

Lymphoma cells represent a mammalian cell system that has been extensively used for GPI-anchor analysis. To test for YW3548 activity in these cells, cultures were labeled with [$^{3}$H]*myo*-inositol for 24 h in the presence of tunicamycin and YW3548, followed by lipid extraction and TLC analysis (Figure 8B). As a marker for the mouse $Man_2$ intermediate, we also labeled SIA-B mutant cells which are defective in the addition of the third mannose residue to the GPI core. The structure of the accumulated intermediate differs from the yeast intermediate by the presence of a side chain ethanolamine which was shown to be attached to the Man-GlcN-PI intermediate before the addition of the second core structure mannose in lymphoma cells (Kinoshita and Takeda, 1994). We found that YW3548 caused the accumulation of an intermediate structure in wild type cells that comigrated with the intermediate that was accumulated in SIA-B cells. Thus, YW3548 appears to affect the same biosynthetic step as does the SIA-B mutation, namely the addition of the third mannose in an α-1,2 linkage onto the GPI core. YW3548 therefore displays the same inhibitory activity in lymphoma cells as in yeast cells.

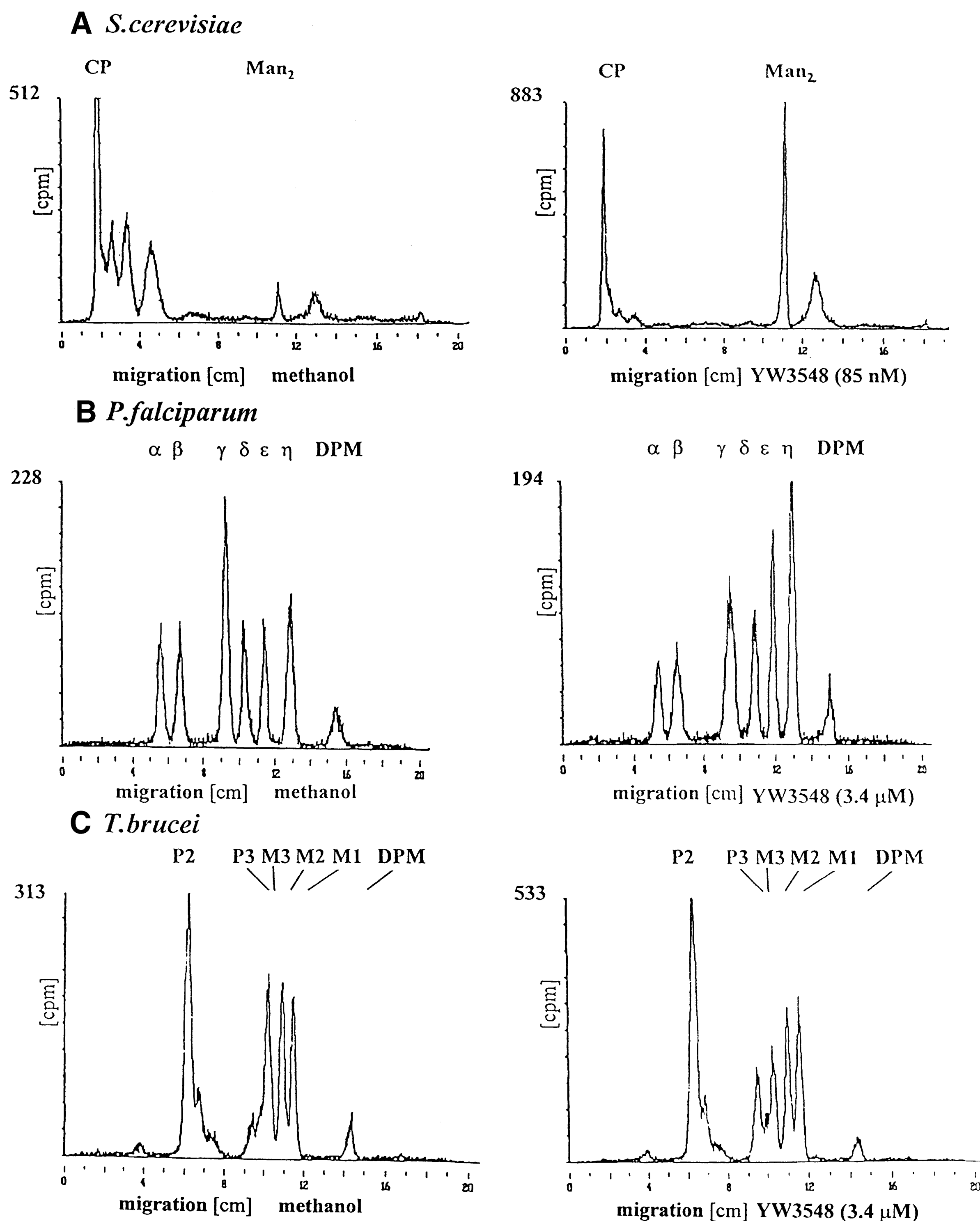


**Fig. 7.** Effect of YW3548 in protozoal systems.The effect of YW3548 on *in vitro* GPI-synthesis was analyzed from yeast (**A**), *T.brucei* (**B**) and *P.falciparum* (**C**). After a 60 min labeling, total lipids were extracted, separated by TLC. The signal was analyzed using a TLC scanner. Known intermediates are indicated from all systems: (A) CP complete precursor, $Man_2$ ($Man_2$-GlcN-PI) (B): α (EthN-P-$Man_4$-GlcN-acylPI), β (EthN-P-$Man_3$-GlcN-acylPI), γ ($Man_4$-GlcN-acylPI), δ ($Man_3$-GlcN-acylPI), ε ($Man_2$-GlcN-acylPI), η ($Man_1$-GlcN-acylPI), DPM (dolicholphophatemannose). (C): P2 (GPI-anchor precursor P2), P3 [GPI-anchor precursor (acylP2), M3 ($Man_3$-GlcN-PI), $M_2$ ($Man_2$-GlcN-PI), M1 mixture $Man_3$-GlcN-acylPI and $Man_1$-GlcN-PI].

## Discussion

We have screened natural compounds for inhibitors of GPI-anchor synthesis in yeast. This approach led to the identification and isolation of the terpenoid lactone YW3548. YW3548 blocks the addition of the third mannose in an α-1,2 linkage to the GPI-intermediate $Man_2$-GlcN-PI and its activity appears to be highly specific for

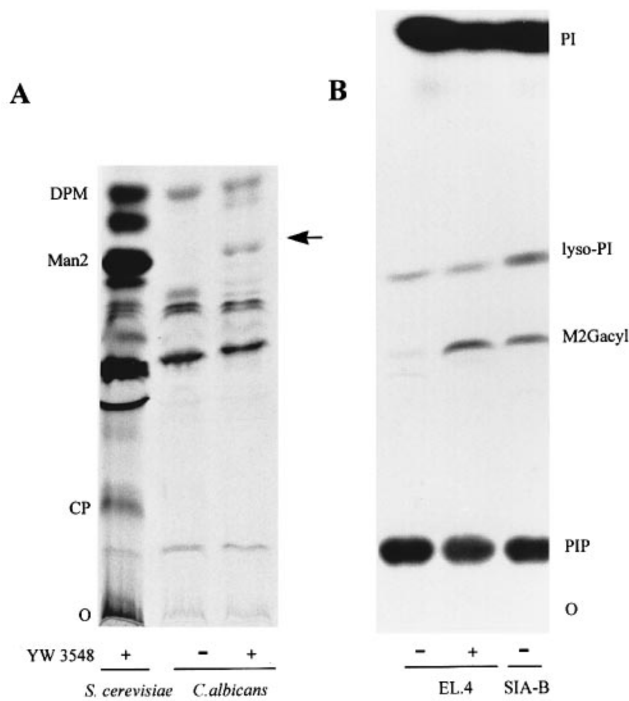


**Fig. 8.** Effect of YW3548 on other systems. Membranes from *C.albicans* were incubated with [$^{14}$C]GDP-Man for 60 min in the presence of 170 nM YW3548 or methanol. Lipids were extracted and analyzed by TLC (**A**). An *in vitro* reaction of *S.cerevisiae* (in the presence of YW3548) was run as standard. The arrow indicates the intermediate accumulated in the presence of YW3548. Lymphoma wild type (EL.4) or mutant cells (SIA-B) were labeled *in vivo* with [$^3$H]*myo*-inositol for 24 h in the presence of tunicamycin. Lipids were extracted and analyzed by TLC. O, origin; PI, phosphatidylinositol; PIP, phosphatidylinositolphosphate; M2Gacyl, $Man_2$GlcNacylphosphatidylinositol.

GPI-anchor synthesis. Mannose residues in α-1,2 linkage are found in the core structure and in outer chains of *N*-linked oligosaccharides, in *O*-linked oligosaccharides and in mannosylated inositol phosphorylceramides. In a mutant affecting an α-1,2 mannosyltransferase activity in core *N*-linked oligosaccharide synthesis, hypoglycosylated CPY was observed (Burda *et al.*, 1996). In our study, the molecular weights of the ER forms of CPY and Gas1p were unaffected by YW3548 (Figure 2A and B). In addition, mannosylated inositol phosphorylceramide synthesis occurred normally in the presence of the inhibitor (Figure 4). We observed, on the other hand, that upon incubation with YW3548, invertase was glycosylated to a slightly greater extent. Since most of the about 60 GPI-anchored proteins predicted from the yeast genome sequence are glycoproteins, a block in GPI-anchoring and therefore their transport to the Golgi might cause a higher availability of GDP-mannose for *N*-glycosylation in the Golgi or less competition for the enzymes that add outer chains in the Golgi. Consistent with this explanation, a similar increase in the molecular weight of invertase has been observed previously in a mutant strain defective in GPI-anchor attachment (Hamburger *et al.*, 1995).

While YW3548 blocks the addition of the third mannose to the GPI core in both *C.albicans* and in lymphoma cells, GPI-anchor synthesis is not affected in parasitic protozoa like *T.brucei*, *P.falciparum*, *P.primaurelia* and *T.gondii* even using 40× the concentration of YW3548 giving complete inhibition in yeast. Although the overall structure of the GPI-core glycan is conserved between parasitic protozoa, yeast and mammalian cells, our data suggest that the GPI-biosynthetic machinery might be different between these species.

The third mannosyltransferase of the GPI-anchor adds a mannose residue from dolichol-*P*-mannose to the glycolipid $Man_2$-GlcN-acylPI in an α-1,2 linkage. We postulate that the lactone ring of YW3548 mimics one of the natural substrates of this α-1,2 mannosyltransferase. Most likely, it does not mimic dolichol-*P*-mannose because this substrate is used for several mannosylation reactions in the cells which are not inhibited by YW3548. It is more likely that YW3548 resembles the GPI-anchor precursor substrate or a corresponding transition state of the reaction. It is conceivable that this highly hydrophobic molecule does so by partially inserting into the lipid bilayer and binding to the mannosyltransferase.

There are general differences between the protozoan GPI-anchors and those from mammalian and yeast cells which could account for the observed species-specific defect. During GPI-precursor synthesis, the acylation of the inositol ring on the 2 position appears to be an obligatory step in mammalian cells as well as in yeast (Costello and Orlean, 1992; Hirose *et al.*, 1992; Puoti and Conzelmann, 1993). Furthermore, it has been shown that the acyl chain is a palmitate in yeast and mammals. In the bloodstream form of *T.brucei*, acylation (palmitate) of position 2 or 3 of the inositol ring can occur, but does not represent an obligatory step in the synthesis pathway (Güther and Ferguson, 1995). In *P.falciparum*, in contrast, protein-bound GPI-anchors have been shown to be myristoylated on the inositol ring (Gerold *et al.*, 1996), although the position has not yet been determined. It is conceivable that the presence or absence of the additional acyl chain or its length determines the presentation of the GPI-intermediate to the GPI α-1,2 mannosyltransferase. The inhibitor could mimic this presentation better in yeast and mammalian cells than in protozoa. It is also possible that the susceptibility of this transferase to the inhibitor is distinct and that the GPI-anchoring machinery has not been conserved during evolution. While several yeast and mammalian genes encoding for components of the GPI biosynthetic machinery have been cloned, apart from the partial purification of de-*N*-acetylase (Milne *et al.*, 1994), no data on GPI-biosynthetic enzymes of protozoa are available and one has to await the cloning of protozoan GPI-biosynthetic enzymes to investigate this further.

GPI-synthesis is essential for all eukaryotic organisms tested thus far (Leidich *et al.*, 1994, Kawagoe *et al.*, 1996) and therefore, a medically relevant GPI-synthesis inhibitor would have to be species-specific. Our data on the novel compound, YW3548, suggest that this may be possible. This compound is clearly species-specific suggesting that there are exploitable differences between GPI-synthesis in protozoa and mammals. Unfortunately, YW3548 specifically affects GPI-synthesis in yeast and mammalian cells, without affecting protozoa, but these results suggest that a complementary inhibitor might exist or could be synthesized that has activity in protozoa without detrimental effects on mammalian cells. In the meantime, YW3548 represents a useful tool to inhibit GPI-synthesis

in cultured yeast and animal cells to further elucidate the role of GPI-anchors in these systems.

## Materials and methods

### Strains and growth conditions

For this study, the *S.cerevisiae* strains RH448 (*Mat***a** *his4 leu2 ura3 lys2 bar1*) and C4 (*Mat***a** *leu2-3,112 ura3-52 pmi40*, provided by A.Conzelmann, Fribourg, Switzerland) were used. A wild type strain of *C.albicans* (SC5314) was obtained from J.Ernst, Düsseldorf, Germany. Precultures of *S.cerevisiae* and *C.albicans* were grown to saturation in complete medium (2% glucose, 1% yeast extract, 2% peptone, 40 mg/l adenine and uracil) and used to inoculate overnight cultures. For metabolic labeling, overnight cultures were grown at permissive temperature in SDYE (0.2% yeast extract, 0.67% yeast nitrogen base without amino acids, 2% glucose with the addition of the required nutrients) to exponential growth phase ($0.5–2\times10^7$ cells/ml).

### Screening and Western blot analysis

For the screening of natural compounds, wild type cells (RH448) in logarithmic growth phase were incubated with 1/50 volume of methanolic extracts from bacteria or fungi for 90 min at 30°C. Cells were lysed in 250 µM NaOH, 0.5% 2-mercaptoethanol, followed by TCA precipitation of total protein. The TCA precipitates were washed with acetone, dried and resuspended in Laemmli sample buffer. Total protein was separated by SDS–PAGE and transferred to nitrocellulose. The filters were blocked with 2% milk in PBS and decorated with polyclonal antiserum directed against Gas1p. Anti-rabbit IgG coupled to peroxidase (Sigma) was used as secondary antibody and detected using the ECL kit (Amersham Int. Amersham, UK).

### Halo assay for killing activity

To test for killing activity of a compound on a yeast lawn, $1–5\times10^6$ YKKB-13 cells (Matα, *ura3*, *leu2*, *his3*, *trp1*, *lys2*, *sts1*Δ*::TRP1*, provided by Karl Kuchler, Vienna, Austria) were included into the plates of complete medium (2% glucose, 1% yeast extract, 2% peptone, 0.8% agar, 40 mg/l adenine and uracil). 4 µl of the compound to be tested was spotted onto the plate which was incubated overnight at 30°C.

### Radiolabeling and immunoprecipitations

Pulse–chase labeling and analysis of immunoprecipitates were done as described previously (Horvath *et al.*, 1994). Briefly, cells were grown overnight in SDYE (2% glucose, 0.2% yeast extract, 0.67% yeast nitrogen base, required amino acids), harvested and washed with SD* (2% glucose, 0.67% yeast nitrogen base and the required nutrients). $2.5\times10^7$ cells/time point were resuspended in 1 ml SD*, preincubated for 10 min with inhibitors, metabolically labeled with TRANS $^{35}$S-label (Dupont de Nemours, Germany) and chased as indicated with 0.3% methionine, 0.3% cysteine, 0.3 M $(NH_4)_2SO_4$. Aliquots were taken, $NaN_3$ and NaF were added to 10 mM final concentration, cells were lysed by vortexing with glass beads in TEPI (100 mM Tris–HCl, pH 7.5, 10 mM EDTA, proteinase inhibitors: 1 mg/ml pepstatin, 1 mg/ml leupeptin, 1 mg/ml antipain). The lysates were boiled in the presence of 1% SDS for 5 min, centrifuged for 15 min in an Eppendorf centrifuge, 5 ml TNET (100 mM Tris–HCl pH 8, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100) was added to the supernatant, and the extract was incubated with antiserum and protein A–Sepharose (Pharmacia, Uppsala, Sweden) for 3 h at RT. The immunoprecipitate was collected and analyzed by SDS–PAGE with subsequent exposure and quantitation of the gel on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Invertase secretion assays were performed as described (Kübler *et al.*, 1994).

### Labeling of GPIs in vitro and in vivo

For the *in vitro* GPI-synthesis system, total yeast membranes were prepared from logarithmically growing wild type strains. Cells were washed in cold 50 mM KPhosphate buffer pH 7.5, they were converted into spheroplasts in PD buffer [50 mM KPhosphate pH 7, 5 mM DTT, 1 M sorbitol, protease inhibitors (1 mg/ml pepstatin A, 1 mg/ml antipain, 1 mg/ml leupeptin, all purchased from Sigma)] using lyticase (Schimmöller and Riezman, 1993) and osmotically lysed by the addition of PD buffer without sorbitol. Membranes were pelleted by centrifugation in a TFT 70.38 rotor (Kontron, Switzerland) at 28 K. The membranes from $2\times10^7$ cells were resuspended in 1 ml PD buffer without sorbitol, 20% glycerol and could be stored for at least 3 months at –80°C. For *in vitro* labeling experiments, a protocol described previously was modified (Costello and Orleans, 1992). In brief, 25 µl of membranes were incubated for 5 min with GPI buffer (1 mM ATP, 1 mM GTP, 1 mM coenzyme A, 20 µg/ml tunicamycin, 10 µM nikkomycin, 0.5 mM UDP-GlcNAc, 150 µg/ml dolicholphosphate, 100 mM Tris–HCl pH 7.5, 1 mM EGTA, 3 mM $MgCl_2$, 0.5 mM $MnCl_2$) in a 100 µl reaction volume in the absence or presence of YW3548 before the addition of 7.5 µCi [$^{14}$C]GDP-Man (DuPont de Nemours, Germany ). After 60 min incubation at 30°C, total lipids were extracted by the addition 660 µl of $CHCl_3$:$CH_3OH$ (1:1). The lipids were dried, desalted by phase partitioning between *n*-butanol and $H_2O$ and analyzed by thin layer chromatography, followed by fluorography. TLC plates were routinely developed in $CHCl_3$:$CH_3OH$:$H_2O$ (10:10:3) or in $CHCl_3$:$CH_3OH$:25% $NH_3$:1 M $(NH)_4OAc$:$H_2O$ (180:140:11:8:21). If the [$^{14}$C]UDP-GlcNAc was used, 0.5 mM UDP-GlcNAc was replaced by 0.5 mM GDP-Man. The same protocol was used for GPI *in vitro* synthesis with *C.albicans* membranes.

Membranes for *in vitro* labeling experiments with [$^3$H]GDP-Man in *T.brucei* were prepared as described (Masterson *et al.*, 1989). Membranes were treated with 0.2 µg/ml tunicamycin (pretreatment of 30 min before membrane preparation and of 15 min before *in vitro* labeling). Membranes were supplemented with 1 mM ATP, 1 mM coenzyme A and 1 mM UDP-GlcNAc and preincubated for 15 min with YW3548 in 100 µl final volume before the addition of 1 or 2 µCi [$^3$H]GDP-Man. After 60 min incubation at 37 °C, lipids were extracted by adding 660 µl $CHCl_3$:$CH_3OH$ (1:1) after 1 min sonication and 15 min incubation at RT, lipids were desalted by phase partitioning between *n*-butanol and $H_20$ and analyzed by TLC. The signal was evaluated using a TLC-scanner (Berthold LB2842). Membranes from *P. falciparum* were freshly prepared and analyzed as described (Gerold *et al.*, 1994).

*In vivo* [$^3$H]mannose labeling using the *pmi40* mutant strain were performed as described (Sipos *et al.*, 1994). In brief, *pmi40* cells were grown overnight in SDCU medium (2% glucose, 1% peptone, 0.67% yeast nitrogen base, 0.1% mannose, supplemented with 40 mg/l uracil. $4\times10^7$ cells were resuspended in SPCU medium (0.1% glucose, 2% pyruvate, 0.67% yeast nitrogen base and the required nutrients), preincubated at non-permissive temperature for 20 min and labeled with 25 µCi [$^3$H]mannose for 30 min. The labeling was stopped by the addition of 10 mM NaF, 10 mM $NaN_3$ and lipids were extracted with $CHCl_3$:$CH_3OH$:$H_2O$ (10:10:3). Lipids were desalted by phase partitioning between *n*-butanol and 0.1 mM EDTA, 5 mM Tris–HCl, pH 7.5 and analyzed by TLC using $CHCl_3$:$CH_3OH$:$H_2O$ (10:10:3) as solvent.

### [$^3$H]myo-inositol labeling of proteins

Wild type cells (RH448) were grown overnight in SDYE medium. $5\times10^7$ cells were washed twice in SD-inositol, resuspended in 500 µl SD-inositol, and preincubated with methanol or 5 µg/ml YW3548 for 10 min before the addition of 15 µCi [$^3$H]*myo*-inositol. Cells were labeled for 30 min. A total protein extract was prepared by lysing the cells in 250 µM NaOH, 0.5% 2-mercaptoethanol and TCA precipitation. The precipitate was washed with acetone and resuspended in Laemmli protein sample buffer. The proteins were separated by SDS–PAGE (10% gel) and after incubation of the gel in 1 M sodium salicylate, the radioactivity was detected by fluorography.

### Analysis of the accumulated intermediate structure

The *in vitro* accumulated intermediate was scraped off the TLC plate, converted into its neutral glycan by dephosphorylation using HF followed by nitrous acid deamination and reduction. The analysis was done by Dionex HPAEC (Gerold *et al.*, 1994).

### Cultivation of lymphoma cells and [$^3$H]-mannose labeling

Wild type (EL.4, received from B.Imhof, Basel, Switzerland) and mutant cells (SIA-B, kindly provided by A.Conzelmann, Fribourg, Switzerland) cells were maintained in Dulbecco's modified eagle medium containing 10% (v/v) fetal calf serum, glutamine in a humidified atmosphere containing 5% $CO_2$. Labeling with [$^3$H]*myo*-inositol was performed as described (Puoti *et al.*, 1991). In brief, $10^7$ cells were resuspended at $10^6$ cells/ml in inositol-free Dulbecco's modified Eagle's medium. Cells were preincubated with tunicamycin (3 µg/ml) for 60 min and with 17 µM YW3548 or DMSO as conrol for 15 min before labeling the cells for 24 h with 15 µCi [$^3$H]*myo*-inositol. Lipids were extracted with $CHCl_3$:$CH_3OH$:$H_2O$ (10:10:3), desalted by phase separation between *n*-butanol and $H_2O$ and analyzed by TLC using $CHCl_3$:$CH_3OH$:0.25% KCl (55:45:10) as solvent.

### *Growth conditions of Codinea simplex and purification of YW3548*

*Codinea simplex* was grown for 7 days in preculture medium at 24°C [0.1% Bacto Agar, 0.4% Yeast Extract (Gistex-X-II), 2% malt extract (Wander)] and used for inoculation (1:20) into growth medium [0.72% Bacto Yeast Extract (Difco), 2% Corn Starch, pH 5.5]. Cells were harvested after 4 days growth at 21°C. To purify YW3548, mycelia were collected by filtration, blended in methanol, filtered and the methanol extract was evaporated until the methanol was removed. After extraction in acetic acid ester, the ester phase was evaporated and dissolved in methanol. YW3548 was purified by the following column steps: separation on Sephadex LH-20, separation on reversed-phase HPLC (C18) 60–100% acetonitrile gradient, separation on a DIOL column using a toluene:acetic acid ester gradient mix, separation on a DIOL column using an hexane:acetic acid ester gradient mix (Y.Wang, manuscript in preparation). The active fractions were determined by halo assay and by testing for Gas1p maturation by Western blot analysis. Stock solutions were prepared in methanol or DMSO and diluted into cultures or cell extracts at a maximum of 2% methanol final concentration.

The structure of YW3548 was determined by spectroscopic methods including IR, FAB-MS, $^{1}$H-NMR, $^{13}$C NMR (JMOD), COSY, ROESY, HSQ and HMBC (Y.Wang, manuscript in preparation). YW3699 like YW3548 is a natural metabolite of *Codinea simplex*. YW3716 is a hydrolysis product of YW3548, YW3713 results from hydrogenation of YW3548 and YW3711 was generated by acetylation of YW3548 (Y.Wang, manuscript in preparation).

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