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A small molecule with differential effects on the PTS1 and PTS2 peroxisome matrix import pathways

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Summary

The use of small molecules has great power to dissect biological processes. This study presents the identification and characterisation of an inhibitor of peroxisome matrix protein import. A mini-screen was carried out to identify molecules which cause alteration in peroxisome morphology, or mislocalisation of a peroxisome targeted fluorescent reporter protein. A benzimidazole lead compound (LDS-003655) was identified which resulted in reduced GFP fluorescence in peroxisomes and cytosolic GFP accumulation. The effect of the compound was specific to peroxisomes as Golgi bodies, endoplasmic reticulum and the actin cytoskeleton were unaffected even at 25 μM, whereas peroxisome import via the PTS1 pathway was compromised at 100 nM. When seedlings were grown on 25 μM LDS-003655 they displayed morphology typical of seedlings grown in the presence of auxin, and expression of the auxin reporter DR5::GFP was induced. Analysis of a focussed library of LDS-003655 derivatives in comparison with known auxins led to the conclusion that the auxin-like activity of LDS-003655 is attributable to its *in situ* hydrolysis giving rise to 2,5-dichlorobenzoic acid, where as the import inhibiting activity of LDS-003655 requires the whole molecule. None of the auxins tested had any effect on peroxisome protein import. Matrix import by the PTS2 import pathway was relatively insensitive to LDS-003655 and its active analogues, with effects only seen after prolonged incubation on high concentrations. Steady state protein levels of PEX5, the PTS1 import pathway receptor, were reduced in the presence of 100 nM LDS-003655, suggesting a possible mechanism for the import inhibition.

Introduction

Peroxisomes are virtually ubiquitous eukaryotic organelles. They can arise *de novo* from the endoplasmic reticulum (ER) membrane, but contain a dedicated protein import apparatus composed of proteins collectively called 'peroxins' (abbreviated PEX) within their single bilayer boundary membrane (Lanyon-Hogg *et al.* 2010). Peroxisome matrix proteins and membrane proteins are imported post translationally after synthesis in the cytosol. With a few exceptions, matrix proteins are targeted by one of two targeting signals. The majority of matrix proteins are targeted by a carboxy-terminal tripeptide, the prototype of which is the sequence serine-lysine-leucine or SKL and is termed the PTS1 sequence. PTS1 proteins are recognised by a cytosolic receptor protein, PEX5, which accommodates the PTS within a binding pocket formed by two groups of three TPR repeats (Gatto *et al.* 2000). The nature of the molecular interaction allows some variants of the PTS to be accommodated with differing affinity provided that the amino acids possess side chains that conform to the consensus of small -basic-hydrophobic (Maynard *et al.* 2004). Upon binding a PTS1 containing cargo protein, PEX5 interacts with the docking peroxins PEX14 and PEX13 on the peroxisome membrane. In a step which is still poorly characterised but may involve oligomers of PEX5 and PEX14 forming a transient pore (Meinecke *et al.* 2010), the cargo is translocated and unloaded from PEX5. The receptor is then monoubiquitinated on a unique amino-terminal cysteine residue by the E2 ligase PEX4 (or UBC5a/b/c in mammals (Grou *et al.* 2008)) and the E3 ligase PEX12 (Platta *et al.* 2009) and

recycled from the membrane by the AAA (ATPases Associated with diverse cellular Activities) proteins PEX1 and PEX6 (Platta *et al.* 2005). As well as monoubiquitination on a cysteine, PEX5 can also undergo polyubiquitination on two lysine residues close to the amino-terminus. Polyubiquitination appears to act as a signal for PEX5 degradation by the proteasome and is readily detected in yeast mutants blocked in recycling, thus it is believed to be a quality control mechanism that acts to remove non-functional PEX5 from the membrane.

The other matrix targeting signal is an amino-terminal nonapeptide termed PTS2, which is recognised by the soluble receptor PEX7. To function in import, PEX7 requires co-receptor(s) to allow docking at the peroxisome membrane. In mammals and plants, PEX5 acts as the co-receptor for PEX7. Less is known about the mechanism of import of PTS2 proteins, but mutants in the docking and recycling peroxins block both PTS1 and PTS2 protein import suggesting the processes are linked at some level. In *Saccharomyces cerevisiae,* which uses Pex18p and Pex21p as co-receptors for Pex7p, Pex18p has been shown to be ubiquitinated and rapidly degraded (Purdue and Lazarow 2001).

Much of the current knowledge of peroxisome protein import is based on studies from *S. cerevisiae*, due to the ease with which genetic mutations can be identified and characterised because peroxisomes are dispensable for growth on glucose. However peroxisomes are essential for of the correct development and ultimately viability of multicellular organisms. Genetic disorders of peroxisome assembly, the peroxisome biogenesis disorders or PBDs, are lethal

in humans, although fibroblasts from patients can be cultured and studied (Wanders and Waterham 2005). Similarly plants with mutations in peroxisome assembly also have severe, sometimes lethal phenotypes (Boisson-Dernier *et al.* 2008, Sparkes *et al.* 2003). A further challenge to deciphering the mechanism of peroxisome protein import is the fragile nature of peroxisomes, which makes the type of biochemical *in vitro* import reactions routinely used to study mitochondrial, chloroplast or ER transport possible but challenging.

The use of small molecules has proved valuable in dissecting protein trafficking both *in vitro* and *in vivo*. For example, ionophores have been widely used to manipulate membrane potential in the study of protein transport across mitochondrial (Pfanner and Neupert 1985) and thylakoid (Klosgen *et al.* 1992) membranes. Brefeldin A, an inhibitor of the GDP exchange factors of some small GTPases (Geldner *et al.* 2003) and Wortmannin, a PI3 kinase inhibitor (Wipf and Halter 2005) have found extensive use in studies of the secretory pathway. However, until now there have been no specific inhibitors of peroxisomal protein import that can assist in the study of this organelle.

Small molecule probes have a number of attractive advantages, especially for *in planta* studies (reviewed in (Hicks and Raikhel 2009, Kaschani and van der Hoorn 2007)), They can be added at the time of choice, after a viable organism has been produced. The dose, and the length of exposure are directly under the control of the experimentalist, and it may be possible to reverse effects by 'washout' type experiments. If the small molecule is assumed to target a

protein, then it may be active against all members of a protein family, thus circumventing some of the difficulties associated with genetic redundancy. A potential drawback is lack of specificity and off target effects, although careful experimental design and interpretation can help to avoid the worst pitfalls. Although small molecule inhibitors have been used for many years, recent development in diversity oriented chemical synthesis and the technology of screening platforms has opened the possibility to go hunting for molecules with desired biological properties.

The use of chemical based approaches in the study of the complex network of plant hormone interactions has been especially productive. Inhibitors of specific members of the large cytochrome P450 family that is involved in synthesis of the plant hormone brassinolide (De Rybel *et al.* 2009b) and in the catabolism of abscisic acid (Kitahata *et al.* 2005) have led to new tools that can identify novel components of the signalling networks and manipulate hormone responses. Recently, the elusive receptor for ABA was identified using a chemical biology approach. (Park *et al.* 2009). Auxin signalling has also been a productive target for chemical screens. Using an auxin regulated GUS reporter construct, natural products Yokolonide A and B were identified as potent inhibitors of the auxin response (Hayashi et al., 2003) as were a range of simple molecules isolated from a 10,000 compound screen (Armstrong et al., 2004). A screen of 10,000 compounds for the ability to affect gravitropism identified 34 which either inhibited or potentiated the gravitropic response (Surpin *et al.* 2005), four of which also resulted in abnormal endomembrane morphologies. One of these,

Gravicin, was subsequently shown to bind PGP19, an auxin efflux protein (Rojas-Pierce *et al.* 2008). Sortin 1 (Zouhar *et al.* 2004) Endosidin (Robert *et al.* 2008) and Dynasor (Macia *et al.* 2006) have all recently been identified as novel inhibitors of endomembrane trafficking events using this type of approach.

In this paper, we describe the identification of a small molecule inhibitor of peroxisomal protein import via a screen for mislocalisation of a GFP-PTS1 reporter protein. We show that this compound has auxin-like activity at high concentration, but is a very potent inhibitor of PTS1 protein import at sub-micromolar concentrations and that the auxin-response and import defects are completely separable, thus the result of distinct targets. The steady state level of the PTS1 import receptor, PEX5, is low in the presence of 100nM of the compound, suggesting a possible mechanism for import inhibition.

Results

A screen for small molecules affecting peroxisomal processes

A small scale screen was carried out to test the feasibility of this approach to identify candidate inhibitors of peroxisomal protein import. Arabidopsis seedlings which express a fusion between GFP and the last 30 residues of multifunctional protein (MFP2) were germinated and grown on agar medium containing chemicals at 25 μM concentration. The GFP-MFP2 fusion protein is targeted to peroxisomes by the PTS1 pathway (its PTS1 is SRL) (Cutler *et al.* 2000). The 70 compounds used in this screen were a by-product of the screen for compounds affecting the gravitropic response described in (Surpin, Rojas-Pierce, Carter, Hicks, Vasquez and Raikhel 2005). They had all passed the first round screen, but were discarded subsequently for a variety of reasons (Surpin, Rojas-Pierce, Carter, Hicks, Vasquez and Raikhel 2005). Importantly, however, they were all known to be bioavailable to plants. After 6 days growth in the light (16h, 23°C) on medium containing chemical, seedlings were examined by confocal laser scanning microscopy (CLSM) for any alteration in the appearance of peroxisomes compared to controls grown without inhibitor. Three compounds caused abnormal distribution of peroxisomes within cells. Three further compounds were identified that caused peroxisomes to appear fainter than controls, sometimes associated with cytosolic GFP. These compounds are candidates for inhibitors of some aspect of the protein import pathway. Two of these compounds are structurally related and their characterisation will be

presented else where (O'Leary-Steele, Brown, Warriner and Baker, manuscript in preparation). In this study we describe the properties and mode of action of Chembridge compound 6097727 and some structural variants (Figure 1). This compound was initially identified as an inhibitor of the gravitropic response (Surpin, Rojas-Pierce, Carter, Hicks, Vasquez and Raikhel 2005) and re-synthesised and renamed LDS-003655 by us.

Compound LDS-003655 specifically affects localisation of a peroxisome matrix reporter

When Arabidopsis seedlings expressing the GFP-PTS1 reporter protein are germinated and grown in multi-well plates in agar medium containing 0.1% DMSO (control) for 6 days in the light, peroxisomes in the hypocotyl appear as motile fluorescent bodies 1-2 μm in diameter as previously described (Figure 2 panel A, arrows denote peroxisomes). In comparison, seedlings grown in the presence of 25 μM LDS-003655 have reduced fluorescent intensity, which is sometimes below the detection limit compared to control seedlings when viewed using the same settings on a CLSM (Figure 2 panel B, arrows denote faint peroxisomes). Cytosolic fluorescence is also visible, suggesting that GFP is not being efficiently imported into peroxisomes. To confirm the presence of peroxisomes in LDS-003655-treated seedlings and to test whether the general biogenesis of peroxisomes is being inhibited by LDS-003655, we used a reporter construct for the peroxisome membrane. The membrane protein PEX10 fused to YFP is targeted to peroxisomes (Sparkes *et al.* 2005) and its

distribution was unaffected by growth in the presence of 25 μM compound LDS-003655 (Figure 2 C and D). Similar effects of compound LDS-003655 were seen in other tissues examined (Supplementary Figure 1).

To investigate the specificity of the effect of compound LDS-003655 on a peroxisome matrix protein reporter, stably transformed Arabidopsis lines expressing a Golgi marker (sialyl transferase-GFP), an actin marker (GFP-fimbrin1 actin binding domain 2), and an endoplasmic reticulum (ER) marker (GFP-HDEL) were grown as before on DMSO or 25 μM compound LDS-003655. After 6 days the organelles were observed using a CLSM. Golgi bodies in LDS-003655-treated seedlings appeared similar to the control in terms of size, distribution, motility and fluorescent intensity (Figure 3A and B). No cytosolic GFP was detected. This result excludes any effect of the compound on GFP fluorescence, or the production of green fluorescence in the cytosol as the result of an unspecified stress response. The motility of the Golgi provides indirect evidence for normal cellular ATP levels in the treated seedlings, since Golgi move by myosin motors on the actin cytoskeleton. The organisation of actin filaments appeared normal in LDS-003655-treated seedlings (Figure 3C and D) as did the cortical ER, which gave a characteristic reticular structure (Figure E and F). ER fragmentation is a sensitive measure of cellular stress. Thus, LDS-003655-treated seedlings do not display obvious abnormalities of any of the other organelles, nor is there evidence of a general toxicity of the compound. Therefore the cytosolic accumulation of the GFP-PTS1 reporter is consistent with a defect in its transport into peroxisomes.

Compound LDS-003655 has auxin-like effects, but auxins do not inhibit import of peroxisomal proteins

Seedlings grown in the presence of 25 μM LDS-003655 have a very striking growth defect, with a very short root and swollen base of the hypocotyl, and they grow agravitropically (Figure 4B) compared to control seedlings (Figure 4A). This is unsurprising since LDS-003655 passed a primary screen for compounds affecting the gravitropic response (Surpin, Rojas-Pierce, Carter, Hicks, Vasquez and Raikhel 2005) and suggests that LDS-003655 has auxin-like activity since IAA and NAA produce similar seedling morphologies when grown on 25 μM of each compound (Figure 4C and D). The auxin-responsive promoter-reporter DR5::GFP was also up regulated by LDS-003655 (Figure 4G) compared to control seedlings (Figure 4E) and similar to NAA treated seedlings (Figure 4F).

To understand the basis for these observations, the effect of known auxins on peroxisome protein import was investigated. To circumvent the growth abnormality caused by germinating and growing the seedlings on such high concentrations of compound LDS-003655, a transplantation assay was used. Seedlings were germinated and grown for 7 days without compound, then transferred to fresh media containing different concentrations of LDS-003655 for 7 days. Under these conditions seedlings had normal morphology, but a clear import defect could still be seen at 100 nM of LDS-003655. Peroxisomes are

more clearly visible when seedlings are grown in this manner, probably in part because GFP is very stable and it is not possible to differentiate between GFP imported by the peroxisomes before the chemical was applied. Nevertheless, cytosolic GFP is clearly visible in the treated, but not control cells – demonstrating that LDS-003655 is a potent inhibitor of GFP-PTS1 import (Figure 5A and B). The auxins IAA, 2,4-D and NAA were tested at 100 nM using the same assay and no effect on the import of the GFP-PTS1 reporter could be seen (Figure 5C to E). In IAA-treated cells, peroxisomes appeared smaller and more abundant, suggesting IAA may act as a peroxisome proliferator (Figure 5C).

Structure-activity relationship data for LDS-003655 point to distinct modes of action in auxin response and peroxisome protein import

To investigate the molecular basis for the mode of action of LDS-003655 and to determine structure-activity relationships (SAR), a small focussed library of variants was synthesised (Figure 1). We explored simple variation of substituents on the phenyl ring, adding both electron donating and withdrawing groups. In addition, a range of C acylated indole analogues were prepared to determine the structural requirements of the heterocyclic moiety.

Freshly synthesised compound LDS-003655 inhibited import at 100 nM as seen previously (Figure 6B), as did LDS-003658 where the chlorine at the 5 position of the benzene ring has been replaced with an electron withdrawing nitro group

(Figure 6C). Other substitutions reduced or abolished activity (Supplementary Figure 2). The equivalent indole analogues retained some activity when acylated at the indole 3-position. We considered that the amide bond in LDS-003655 (and LDS-003658) may be hydrolytically labile and that activity may be resulting from one of the hydrolysis products. The two component parts of LDS-003655, 2,5-dichlorobenzoic acid and 2-methylbenzimidazole, were tested and found to not have an effect on the localisation of the reporter protein (Figure 6E and F). Furthermore the activity of the stable C-3 acylated indole LDS-003662 in this assay (Figure 6D) suggests that the pharmacophore indeed requires both functional parts of the molecule LDS-003658.

The various analogues were tested for their ability to cause an auxin-like growth phenotype (Figure 7). At 1μM, neither LDS-003655 (Figure 7F) nor the more hydrolytically stable analogue LDS-003663 (Figure 7H) had any effect on seedling growth and appeared similar to control plants (Figure 7A and C). Notably the roots were of normal length. No effect was also caused by growth on 2-methylbenzimidazole (Figure 7I). However, at the same concentration, IAA (Figure 7D), NAA (Figure 7E) and 2,5-dichlorobenzoic acid (Figure 7G) all resulted in a characteristic auxin growth response. These results are consistent with the auxin-like activity of LDS-003655 at high concentration, arising from small amounts of hydrolysis of LDS-003655 to yield to 2,5-dichlorobenzoic acid *in situ*, which has auxin-like activity. This interpretation is substantiated by the finding that compound LDS-003658, which is a similarly potent import inhibitor to LDS-003655 (Figure 6C) but would hydrolyse to produce 5-nitrobenozoic acid

instead of 2,5-dichlorobenzoic acid, does not result in an auxin-like growth response even at 25 μM (Figure 7B).

One way that auxins act is by promoting association between AUX/IAA proteins and F-box proteins of the TIR1 family (TIR1 itself and AFBs1-5)(De Rybel *et al.* 2009a, Tan *et al.* 2007). The F-box proteins form part of an SCF-type E3 ligase which targets the AUX/IAA proteins for polyubiquitination and so degradation in the 26S proteasome. The influence of different auxins on this interaction can be assayed *in vitro* using pulldown assays with epitope-tagged TIR1 and a synthetic peptide from domain II of the Aux/IAA IAA7 (Kepinski and Leyser 2005). NAA at 25 μM is highly effective at promoting the interaction between TIR1 and the target peptide, but neither LDS-003655 or 2,5-dichlorobenzoic acid have an effect in this assay (Supplementary Figure 3). Thus the auxin-like effects of LDS-003655 and 2,5-dichlorobenzoic acid are not the result of their direct binding in the TIR1-Aux/IAA receptor complex.

Compound LDS-003655 and active analogues have differential effects on PTS1 and PTS2 protein import pathways

There is a second import pathway into the peroxisome matrix mediated by PTS2 and PEX7. To investigate the effect of LDS-003655 and its various analogous on this pathway, Arabidopsis seedlings expressing a PTS2 targeted GFP reporter protein consisting of the first 40 amino acids of peroxisomal citrate synthase 3 (CSY3) fused to GFP (Pracharoenwattana *et al.* 2005) were tested

in the transplant assay. In contrast to the effect on the PTS1 reporter, neither LDS-003655 or LDS-003658 inhibited import of the PTS2 reporter at 100 nM (Suplementary Figure 4) and had only a marginal effect at 25 μM after 6 days growth (data not shown). However, after 11 days growth on 25 μM LDS-003655, a PTS2 import defect could be detected in the cotyledons (Figure 8C), but not strikingly in the hypocotyls (Figure 8D). GFP localisation and intensity in seedlings grown on 25 μM of the stable C-3 acylated indole LDS-003663 (Figure 8E and F) was similar to DMSO controls (Figure 8A and B). Thus compound LDS-003655 has a differential effect on the PTS1 and PTS2 matrix protein import pathways.

The steady state levels of the PTS1 receptor, PEX5, and the PTS2 receptor, PEX7, in treated and untreated seedlings were examined by immunoblotting. In LDS-003655-treated seedlings the steady state level of PEX5 was reduced compared to control (DMSO), LDS-003663 or 2,5-dicholorbenzoic acid treated seedlings (Figure 9A). PEX7 levels do not appear to be affected, which fits in with our observation that the PTS2 import pathway is not perturbed at this concentration. This effect was seen in three independent experiments when seedlings were transplanted to media containing 100 nM LDS-003655, and sometimes, although not reproducibly, when seedlings were germinated and grown in the presence of 25 μM LDS-003655. Semi-quantitative RT-PCR was performed on seedlings grown on 100 nM of compound to determine whether LDS-003655 decreases *PEX5* transcript levels. Figure 9B shows that *PEX5* levels are unchanged compared to the DMSO control (and other compounds).

Discussion

In this paper we describe a small molecule (LDS-003655) with a selective inhibitory effect on PTS1 import into plant peroxisomes. This molecule was isolated from a group of compounds that were originally identified as inhibitors of the gravitropic response. We show that at the relatively high concentrations used for screening, LDS-003655 has auxin like activity, explaining its identification in the initial screen. The *in vitro* stability and *in vivo* metabolism of small molecules applied in chemical screens needs to be given careful consideration in the interpretation of the results. Sirtinol was identified as a molecule which caused auxin-like effects on Arabidopsis root and vascular development and up regulated auxin responsive genes (Zhao *et al.* 2003), however, further research established that sirtinol is metabolised *in vivo* to the active 2-hydroxy-1-naphthoic acid which is known to have auxin activity (Dai *et al.* 2005).

In the present case, the auxin response is most likely due to small amounts of hydrolysis of LDS-003655 to release 2,5-dichlorobenzoic acid. The latter compound results in strong stunting of roots at 1 μM compared to the parent compound. Likewise an analogue of LDS-003655 (LDS-003658) which would not yield 2,5-dichlorobenzoic acid upon hydrolysis does not exhibit auxin-like activity even at high (25μM) concentration. Conversely none of the auxins tested showed any inhibitory effect on PTS1 protein import, whereas LDS-

003655 was a potent inhibitor down to 100 nM and the whole molecule rather than the component parts were required for this effect. Thus we conclude that the auxin-like activity and import inhibition are separable and unrelated effects.

SAR studies showed that only limited substitution of the benzene ring was possible to retain activity. Some modification of the heterocycle was possible with the C-3 acylated indole analogues retaining some activity.

LDS-003655 is a specific inhibitor of peroxisomal protein import. Even at high (25μM) concentrations, no effect was seen on the morphology or movement of Golgi and the actin cytoskeleton and ER retained a normal morphology. Interestingly the import of a PTS2 reporter was at least two orders of magnitude less sensitive to LDS-003655 compared to PTS1 protein import and required longer incubation to see an effect. In plants, in contrast to yeast but as is the case in mammals, PEX5 interacts with PEX7 (Nito *et al.* 2002) and mutations in PEX5 fall into two classes, those which affect both PTS1 and PTS2 import (Hayashi *et al.* 2005) and those which affect interaction between PEX7 and PEX5 and therefore affect PTS2 import but not PTS1 import (Woodward and Bartel 2005). This has led to the notion that the PTS2 pathway is dependent upon the PTS1 import pathway.

Conversely, characterisation of a novel mutant allele of *pex7* (*pex7-2*) which had lost the ability to bind both PEX5 and PTS2 cargo, showed that import of a GFP-PTS1 reporter was inhibited, but only in light grown seedlings. In this

mutant PEX5 levels are reduced in both light and dark but much more strongly in the light (Ramon and Bartel 2010). A similar observation for the dependence of the PTS1 import pathway on PEX7 was also seen in the procyclic but not blood stream form of *Trypanosoma brucei* (Galland *et al.* 2007) and *Hansenula polymorpha* mutants in the Pex7p co-receptor, Pex20p, also have reduced levels of PEX5 (Moscicka *et al.* 2007).

Compound LDS-003655 gives the first insight that it may be possible to disrupt the PTS1 pathway specifically. Western blotting showed that PEX5 levels were reduced in LDS-003655-treated seedlings. The data do not establish if the PTS1 import defect is a direct consequence of reduced PEX5 levels, or if PEX5 is reduced as a consequence of a block elsewhere in the import pathway. Studies on yeast have already shown that if PEX5 cannot be recycled from the peroxisome membrane, it is targeted for degradation (Platta, Grunau, Rosenkranz, Girzalsky and Erdmann 2005) and that mutations in both human and Arabidopsis PEX6, one of the AAA ATPases required for PEX5 recycling, result in reduced steady state levels of PEX5 (Dodt and Gould 1996, Zolman and Bartel 2004).

There are a number of interesting questions about the interrelationships between the PTS1 and PTS2 pathways that remain to be unravelled. For example can a single PEX5 molecule simultaneously service both the PTS1 and PTS2 pathways, or are binding of PTS1 cargo and PEX7-PTS2 cargo mutually exclusive? What happens to the PEX5-PEX7 complex after cargo

unloading? Are the two receptors recycled together or do they take separate routes? Genetic approaches are not well suited to examining such dynamic processes. Small molecules with differential effects on PTS1 and PTS2 import may be useful tools to examine these types of questions.

Experimental procedures

Plant materials and growth conditions

Fluorescent fusion proteins were visualised using *Arabidopsis thaliana* lines containing the following constructs: 35S::MFP2-GFP (Cutler, Ehrhardt, Griffitts and Somerville 2000); 35S::PEX10-eYFP (Sparkes, Hawes and Baker 2005); 35S::CSY3-GFP (Pracharoenwattana, Cornah and Smith 2005); 35S::ST-GFP (Saint-Jore *et al.* 2002), 35S::GFP-HDEL (Batoko *et al.* 2000) and 35S::FABD2-GFP (Sheahan *et al.* 2004). A DR5::GFP line was used to determine *DR5* up-regulation (Friml *et al.* 2003).

Seeds were sterilised and stratified in darkness for 48 h at 4℃ before being sown on ½ Murashige and Skoog (MS) media (Ducheva, Haarlem, Netherlands) containing 0.8% plant agar for vertical growth on square Petri dishes or 0.3% plant agar for 24-well microplate growth. All compounds tested were dissolved in DMSO to give 25 mM stock solutions. Stocks were diluted down to either 25 µM or 100 nM with hand hot ½ MS media. Seeds plated onto 25 µM of compound were done so in 24-well microplates and grown for 6 days (unless otherwise stated). Seeds grown on 100 nM of compound were grown vertically on ½ MS media in square plates without compound for 7 days, before being transplanted onto ½ MS media containing 100 nM compound for a further 7 days growth. All plates were incubated in the light (16 h per day) at 23℃.

Confocal laser scanning microscopy

An upright laser scanning microscope (LSM 510; Zeiss, Jena, Germany) with an argon laser and a 63x oil immersion objective was used for confocal imaging. All images were scanned using identical conditions (laser power, gain of photomultiplier tube, pinhole size, and zoom) in relation to the relevant controls.

For imaging GFP fusion proteins, excitation line of an argon ion laser of 488 nm was used with a 505- to 530-nm band pass filter in the single-track facility of the microscope. For imaging PEX10-YFP, excitation line of an argon ion laser of 514 nm was used with a 530- to 600-nm band pass filter in the single-track facility of the microscope.

Post-acquisition image processing was done using the LSM 5 browser (Zeiss) and Adobe Photoshop 9.0 software (Adobe Systems, Mountain View, CA, USA).

Antibodies, polyacrylamide gel electrophoresis and western Blotting

Antibodies against Arabidopsis PEX7 were raised in rabbit by a commercial supplier (Eurogentech) against a peptide CYVWQQGMDPRAS, which corresponds to the native C-terminus of the molecule. Anti-PEX5 (Nito, Hayashi and Nishimura 2002) was a kind gift from Dr Makoto Hayashi and anti-ATPβ

was purchased from Agrisera (Agrisera Antibodies, catalogue number AS05 085).

Total protein was extracted from seedlings according to (Footitt *et al.* 2002) except PMSF and Sigma Protease Inhibitor Cocktail were used at 4 mM and 2% respectively. Protein concentration was determined by the BCA method and 20 µg of total protein from each sample was used for Western analysis. Proteins were separated by standard SDS-PAGE and transferred to nitrocellulose membranes (0.45 µm, Micron Separations, Schleicher&Schuell). Membranes were blocked for 2 hours at room temperature in TBST (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 0.1% v/v Tween 20) containing 5% (w/v) non-fat dry milk, (TBST-milk) then incubated in TBST plus 3% (w/v) BSA (Sigma) containing anti-PEX5 (1 in 1000 dilution). Incubations with primary antibody were carried out overnight at 4 ℃. After four 10-min washes in TBST, membranes were incubated with horseradish peroxidase-conjugated goat-anti-rabbit secondary antibodies (Sigma) for 45 min at 1:5000 dilution in TBST-milk. After four 10-min washes in TBST, membranes were developed by enhanced chemi-luminescence. To assess PEX7 and ATPβ levels, membranes were stripped by incubating in stripping solution (62.5 mM Tris pH 6.7, 2 % SDS, 0.7 % β-mercaptoethanol) at 50 ℃ for 30 min and washed well with TBS before being blocked and re-probed with anti-PEX7 (1 in 1000 dilution) and anti- ATPβ (1 in 5000 dilution).

RT-PCR

Total RNA was isolated using the Qiagen Plant RNeasy Mini Kit in conjunction with the Qiagen RNase-free DNase Set 50 Kit (Westburg, the Netherlands). 200 ng of total RNA was reversed transcribed according to protocol provided with Superscript II reverse transcriptase (Invitrogen Life Technologies, Paisley, UK). 1 µl of cDNA per sample was amplified using KOD DNA polymeragse (Novagen) and primers either specific for *PEX5* (*At*ATf: 5´-AATGGGTGGGCTACTGAGTTCGAGCAG-3´ and *At*ATr: 5´-AGCATTCTCAGGGTTTTTCATAACCTCAGC-3´) or 18S ribosomal RNA (Charlton *et al.* 2005).. The cycling conditions used were: 98 ℃ x 1 min (1 cycle), 98 ℃ x 20 s, 67.3 ℃ x 30 s (40 cycles). Each reaction was analysed after 20, 30 and 40 cycles.

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Figures

Figure 1: Structures of the compounds used in this study.

Figure 2: LDS-003655 inhibits PTS1-mediated import in six day old Arabidopsis seedlings but does not impair peroxisome membrane biogenesis.

GFP-PTS1 fluorescence in hypocotyl cells of seedlings grown for 6 days on ½MS medium (0.3% agar), containing either (**A**) DMSO (0.1%) or (**B**) 25 µM LDS-003655. Arrows denote peroxisomes, which are fainter in (**B**), where cytosolic GFP also appears to accumulate. Peroxisome membrane protein import is not affected as the pattern of PEX10-YFP fluorescence is comparable between hypocotyl cells of seedlings grown for 6 days on ½MS medium (0.3% agar) on (**C**) DMSO (0.1%) and (**D**) 25 µM LDS-003655. Asterisk in (**D**) shows zoomed region of CLSM image containing PEX10-YFP localised to the peroxisome membrane (hence "hoop" pattern of fluorescence). Scale bar = 20 µM.

Figure 3: LDS-003655 has a specific effect on peroxisomes as Golgi bodies, the actin-based cytoskeleton and the ER appear to be unaffected.

Fluorescent pattern of GFP-reporter proteins for (**A** & **B**) Golgi, (sialyltransferase-GFP, arrows denote Golgi bodies), (**C** & **D**) actin (GFP-fimbrin1 actin binding domain 2, arrows denote actin filaments) and (**E** & **F**) the ER (GFP-HDEL, arrows denote ER network). Images are of hypocotyl cells

from 6 day old seedlings which have been grown on ½MS medium (0.3% agar), containing either 0.1% DMSO (**A**, **C** & **E**) or 25 µM LDS-003655 (**B**, **D** & **F**). Scale bar = 20 µM (**A** & **B**), 10µM (**C** & **D**) and 5 µM (**E** & **F**).

Figure 4: LDS-003655 exhibits auxin-like activity.

Seedlings grown on 25 µM LDS-003655 for 7 days exhibit an auxin growth response (A-D). Arabidopsis seedlings (GFP-PTS1 background line) were grown for 7 days on ½MS medium (0.8% agar) containing either (**A**) DMSO (0.004%), (**B**) 25 µM LDS-003655, (**C**) 25 µM IAA or (**D**) 25 µM NAA Scale bar = 1.5 mm. **NAA and LDS-003655 up-regulate the auxin inducible gene DR5::GFP (E-G).** DR5::GFP fluorescence in root cells of seedlings grown for 7 days on ½MS medium (0.8% agar) then transferred for a further 7 days onto ½MS medium containing either (**E**) DMSO (0.004%) or 100 nM of either (**F**) NAA (auxin known to up-regulate DR5) or (**G**) LDS-003655. Scale bar = 20 µM.

Figure 5: LDS-003655 impairs GFP-PTS1 import at 100 nM, while the auxins 2,4-D and NAA have no noticeable effect on import, although IAA appears to proliferate peroxisomes.

GFP-PTS1 fluorescence in root cells of seedlings grown for 7 days on ½MS medium (0.8% agar) then transferred for a further 7 days onto ½MS medium containing either (**A**) DMSO (0.004%) or 100 nM of each of the following compounds (**B**) LDS-003655, (**C**) IAA, (**D**) 2,4-D and (**E**) NAA. Impairment in GFP-PTS1 import can be seen as an accumulation of GFP fluorescence in the cytosol. Scale bar = 20 µM.

Figure 6: The whole LDS-003655 molecule is needed for inhibition of PTS1 import and only limited substitution is possible.

GFP-PTS1 fluorescence in root cells of seedlings grown for 7 days on ½MS medium (0.8% agar) then transferred for a further 7 days onto ½MS medium containing either (**A**) DMSO (0.004%) or 100 nM of each of the following compounds (**B**) LDS-003655, (**C**) LDS-003658, (**D**) LDS-003662, (**E**) 2,5-dichlorobenzoic acid and (**F**) 2-methylbenzimidazole. Impairment in GFP-PTS1 import can be seen as an accumulation of GFP fluorescence in the cytosol. Scale bar = 20 µM.

Figure 7: The auxin-like activity of LDS-003655 results from its hydrolysis to 2,5-dicholobenzoic acid, a molecule with potent auxin activity

Arabidopsis seedlings (GFP-PTS1 background line) were grown for 7 days on ½MS medium (0.8% agar) containing the following: (**A**) 0.1% DMSO, (**B**) 25 µM LDS-003658, (**C**) 0.004% DMSO, (**D**) 1 µM IAA, (**E**) 1 µM NAA, (**F**) 1 µM LDS-003655, (**G**) 1 µM 2,5-dichlorobenzoic acid, (**H**) 1 µM LDS-003663 and (**I**) 1 µM 2-methylbenzimidazole. Scale bar = 1.5 mm.

Figure 8: 25 µM LDS-003655 inhibits PTS2 import at 11 days growth in cotyledons

CYS3-GFP fluorescence in either cotyledons (**A**, **C** & **E**) or hypocotyls (**B**, **D** & **F**) of seedlings grown for 11 days on ½MS medium (0.3% agar) containing

either 0.1% DMSO (**A** & **B**), 25 µM LDS-003655 (**C** & **D**) or 25 µM LDS-003663 (**E** & **F**). Scale bar = 20 µM.

Figure 9: Seedlings transplanted onto 100 nM of LDS-003655 have low levels of PEX5, but PEX7 levels are unaffected.

(**A**) Immunoblot showing PEX5 levels in total protein extracts (20 µg per lane) from 14 day old seedlings grown for 7 days on ½MS medium (0.8% agar) before being transferred for a further 7 days onto ½MS medium containing either DMSO (0.004%) or 100 nM of each of the compounds shown. Blots were stripped and probed with anti-ATPβ to determine loading and stripped again to determine PEX7 levels. (**B**) RT-PCR showing *PEX5* transcript (after 40 PCR cycles) in 14 day old seedlings grown for 7 days on ½MS medium (0.8% agar) before being transferred for a further 7 days onto ½MS medium containing either DMSO (0.004%) or 100 nM of each of the compounds shown.

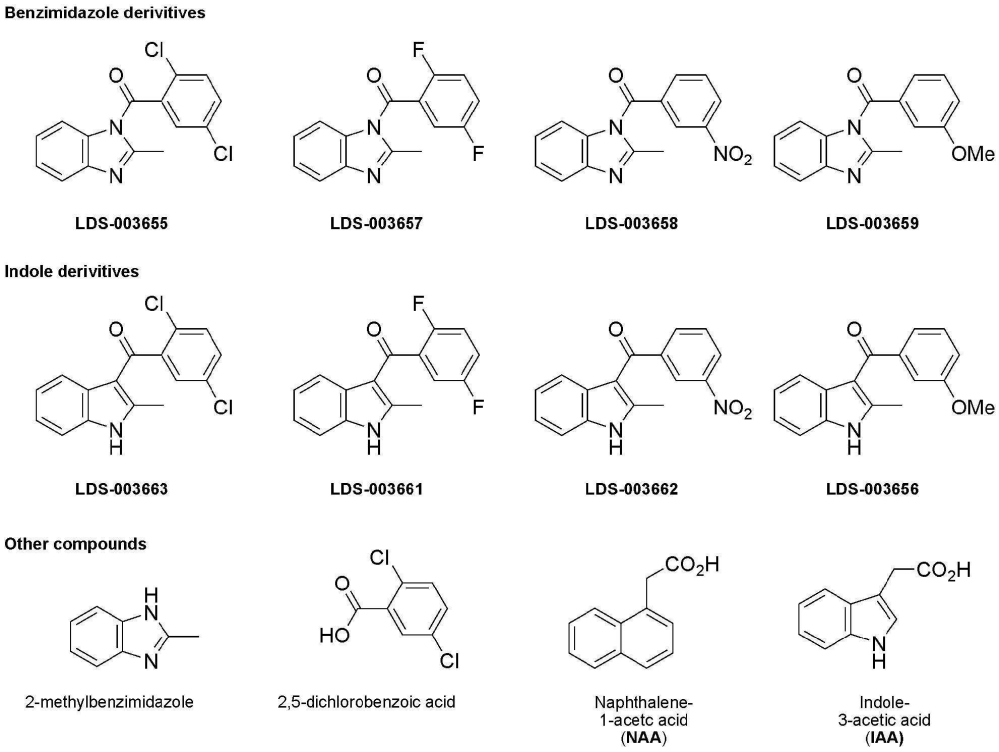
Supplementary figures

Fig S1: Effect of 25 µM LDS-003655 on PTS1-mediated import in cotyledons and roots of Arabidopsis seedlings.

Fig S2: Effect of 25 µM LDS-003655 analogues on PTS1-mediated protein import in roots of Arabidopsis seedlings.

Fig S3: Unlike NAA, LDS-003655 and 2,5-dichlorobenzoic acid do not promote the interaction between TIR1 and IAA7.

Fig S4: Import of CYS3-GFP, a PTS2 containing peroxisomal protein fused to GFP, does not appear to be significantly affected by 100 nM LDS-003655.



Structures of the compounds used in this study
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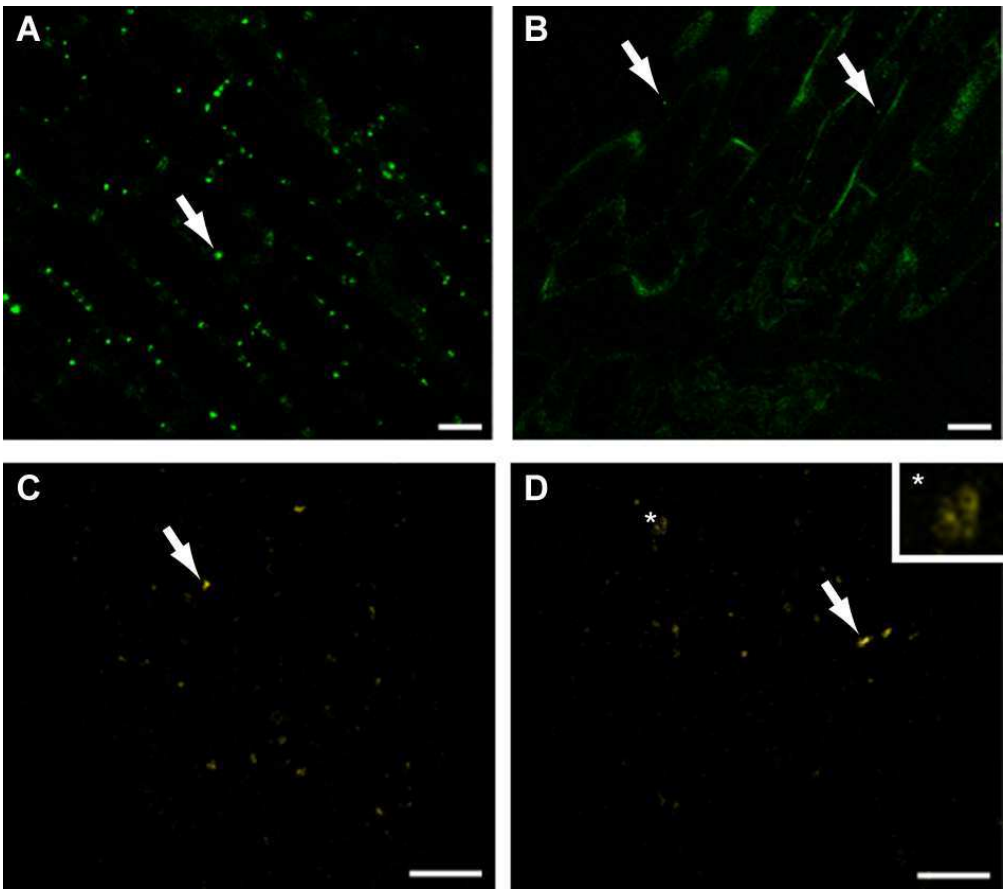

LDS-003655 inhibits PTS1-mediated import in six day old Arabidopsis seedlings but does not impair peroxisome membrane biogenesis
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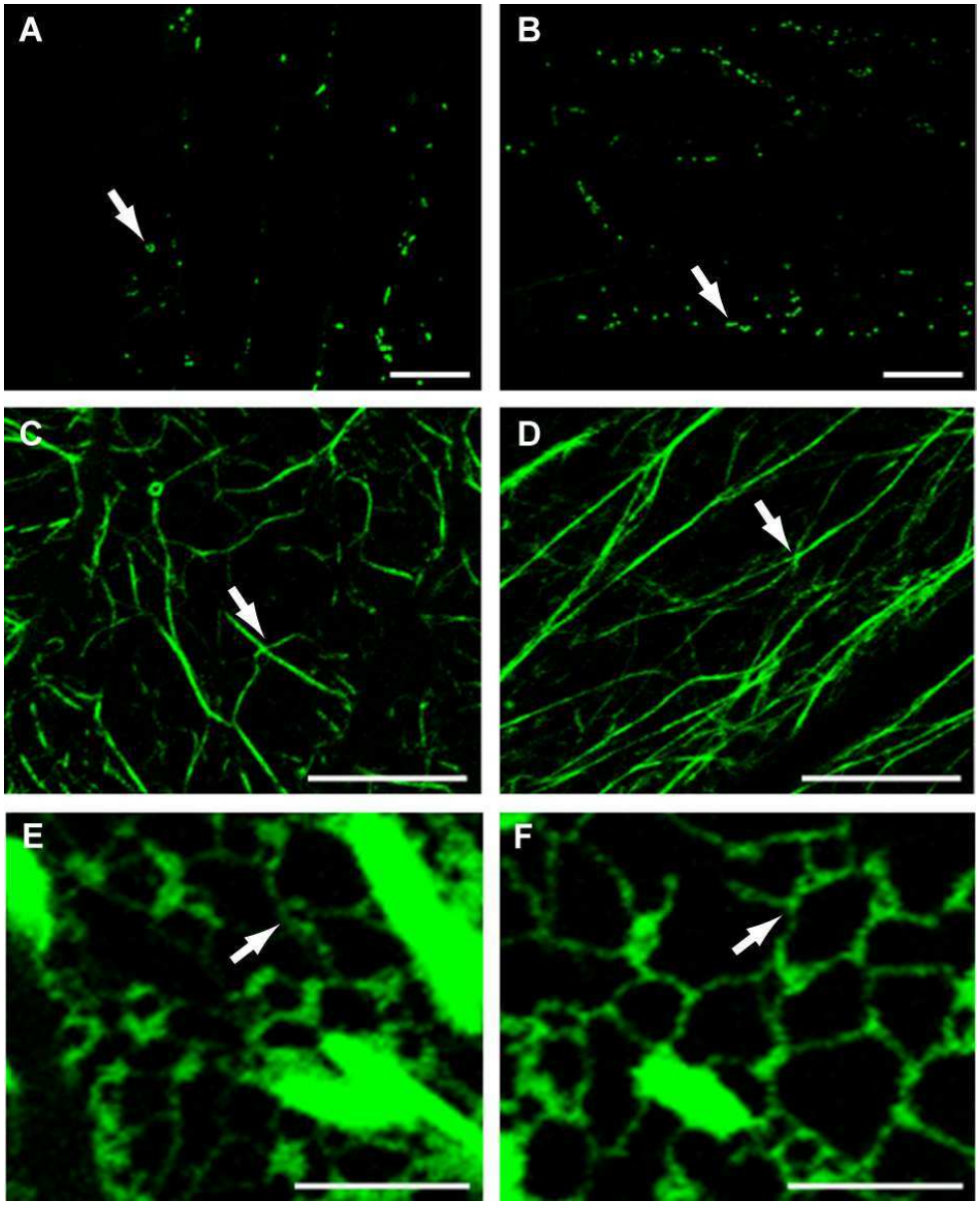

LDS-003655 has a specific effect on peroxisomes as Golgi bodies, the actin-based cytoskeleton and teh ER appear unaffected
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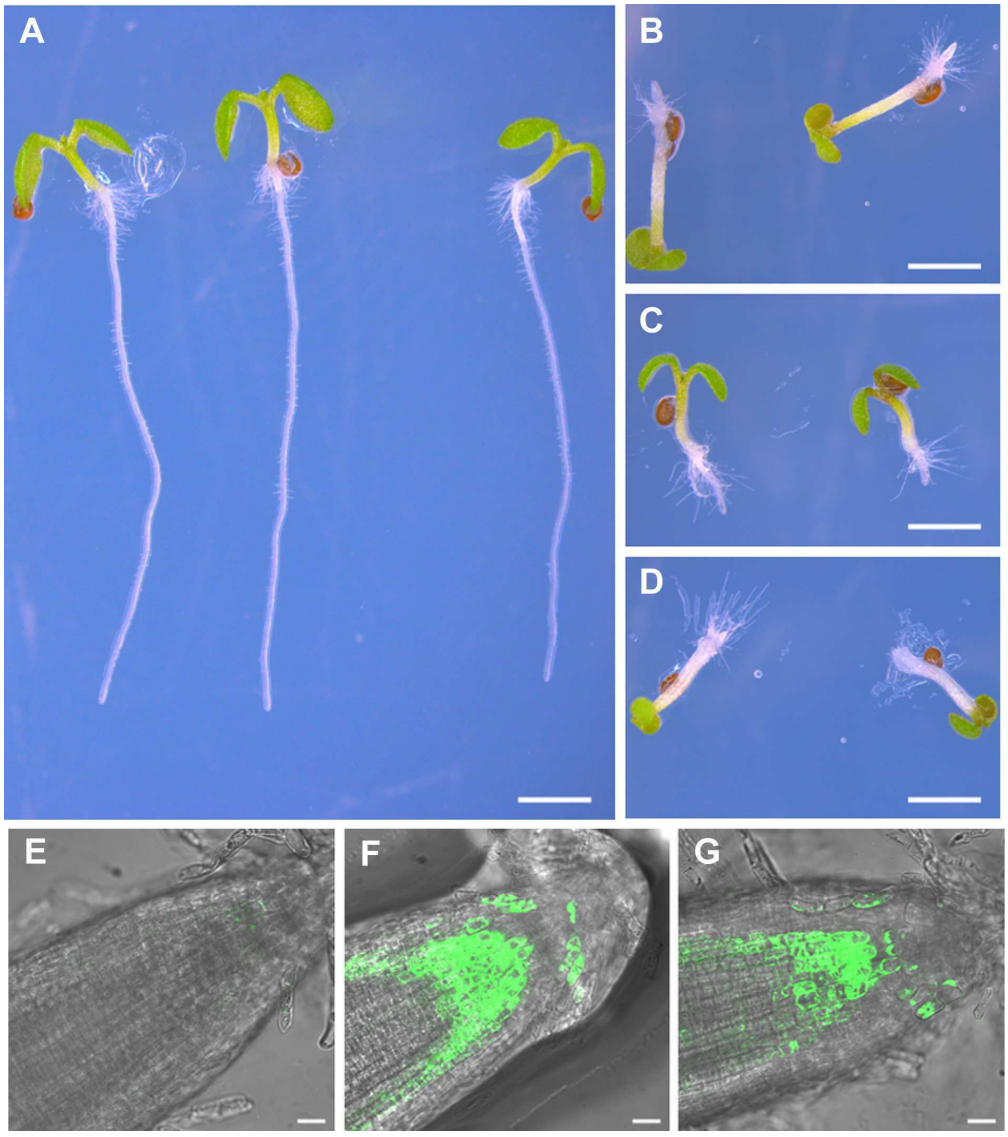

LDS-003655 exhibits auxin-like activity
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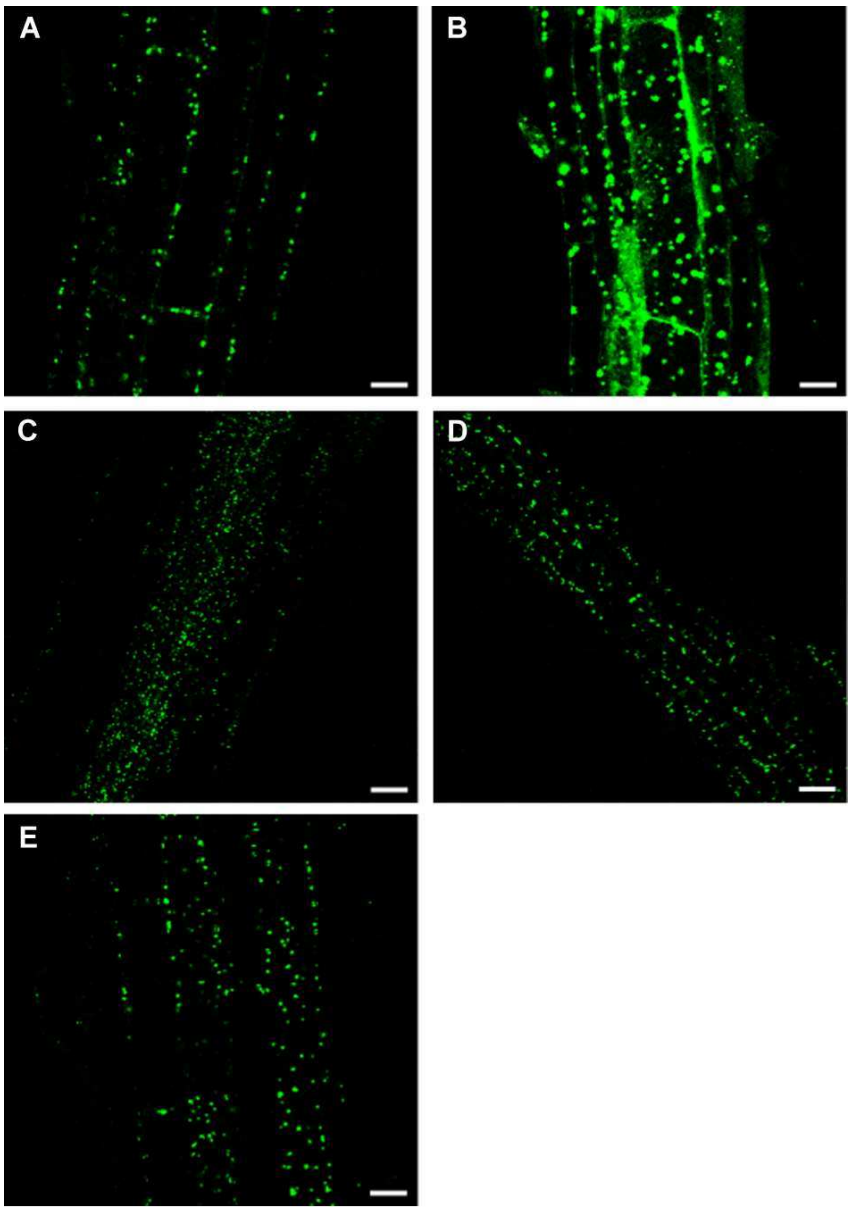

LDS-003655 impars GFP-PTS1 import at 100nM whilst the auxins 2,4-D and NAA have no noticeable effect on import
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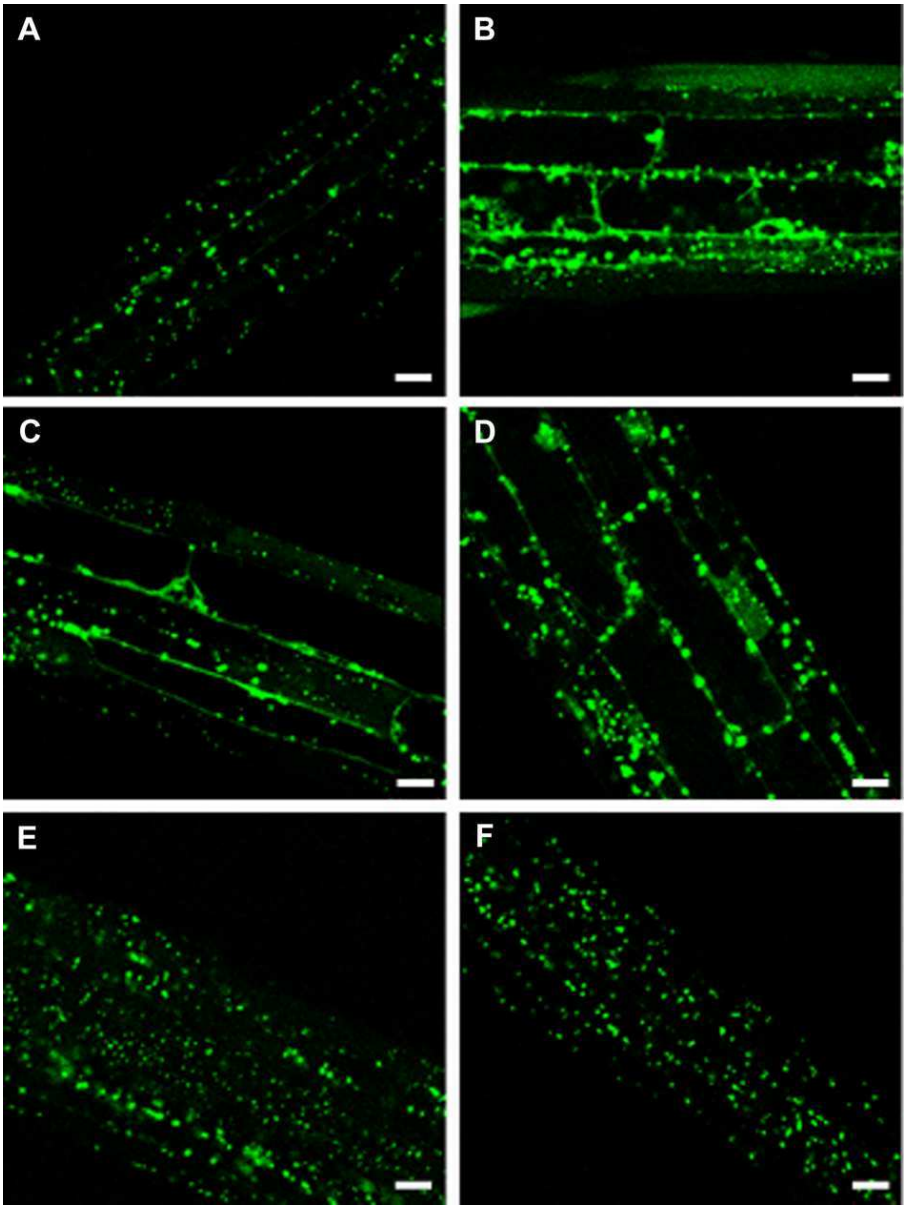

The whole LDS-003655 molecule is needed for PTS1 import inhibition and only limited substitution is possible
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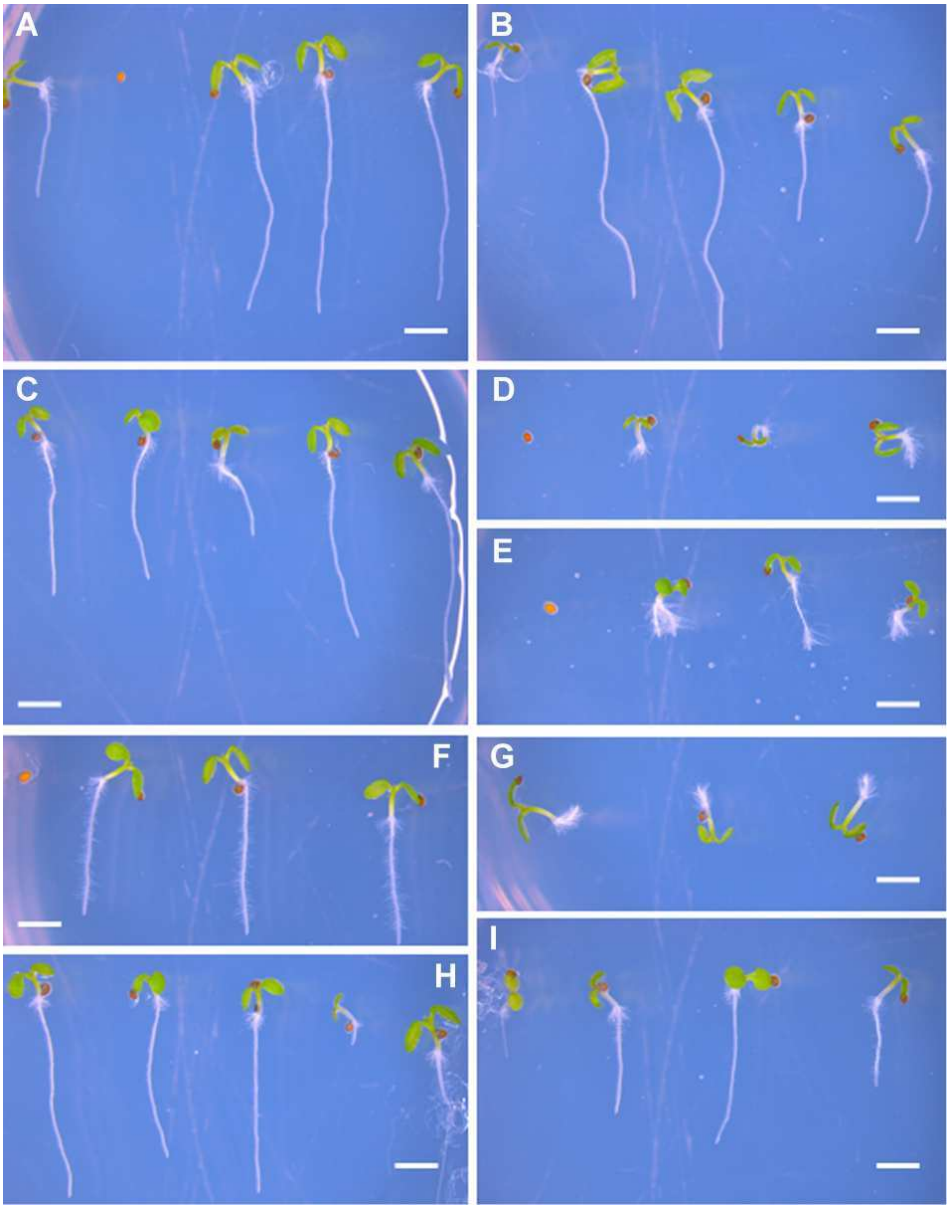

The auxin like activity of LDS-003655 results from its hydrolysis to 2,5-dichlorbenzoic acid, a molecule with potent auxin activity
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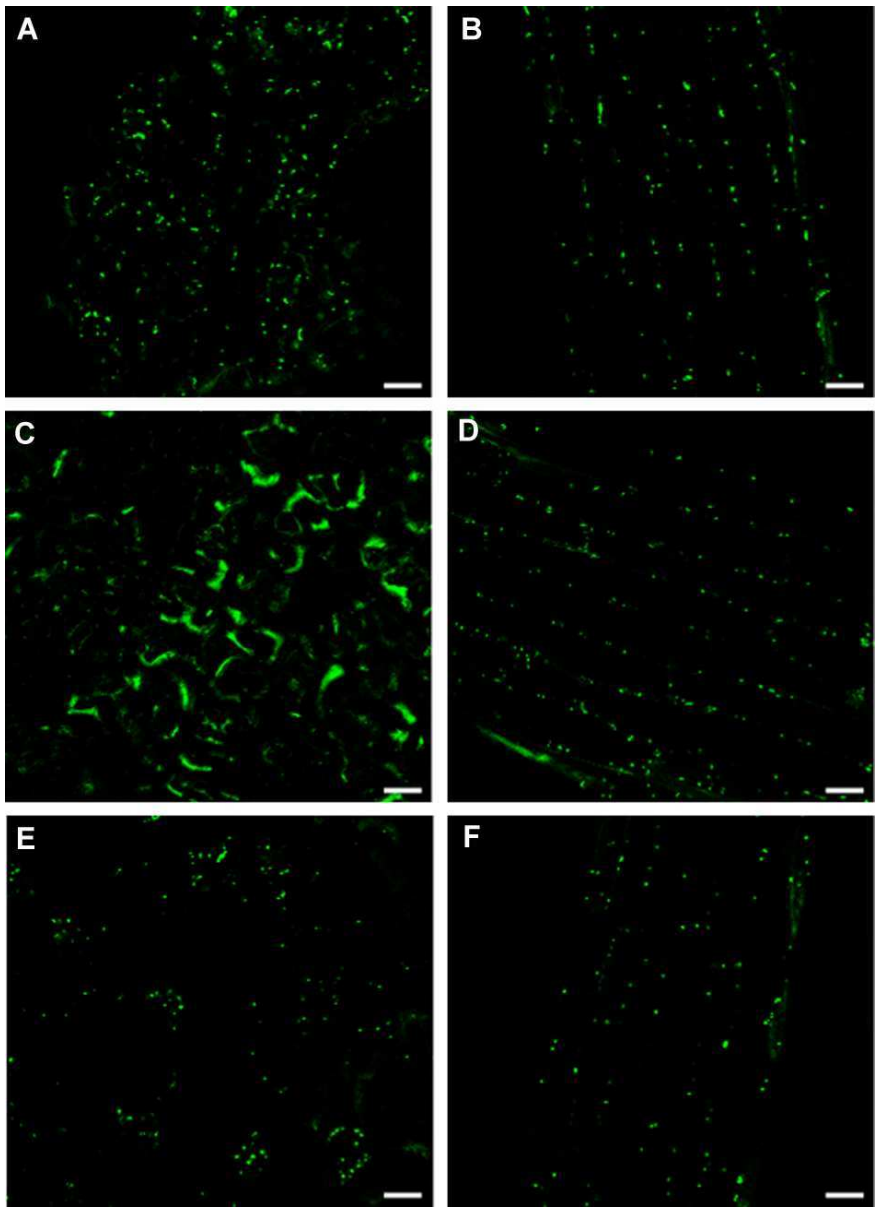

Twenty five micromolar LDS-003655 inhibits PTS2 import at 11 days growth in cotyledons
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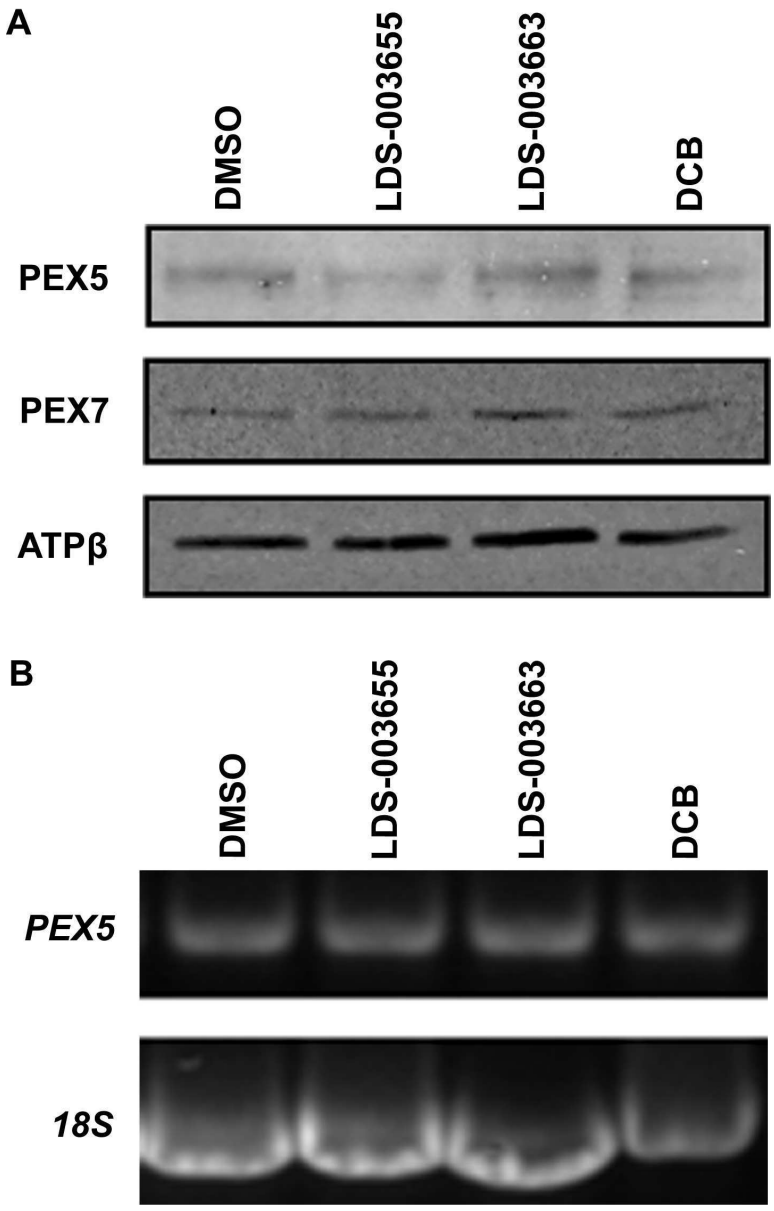
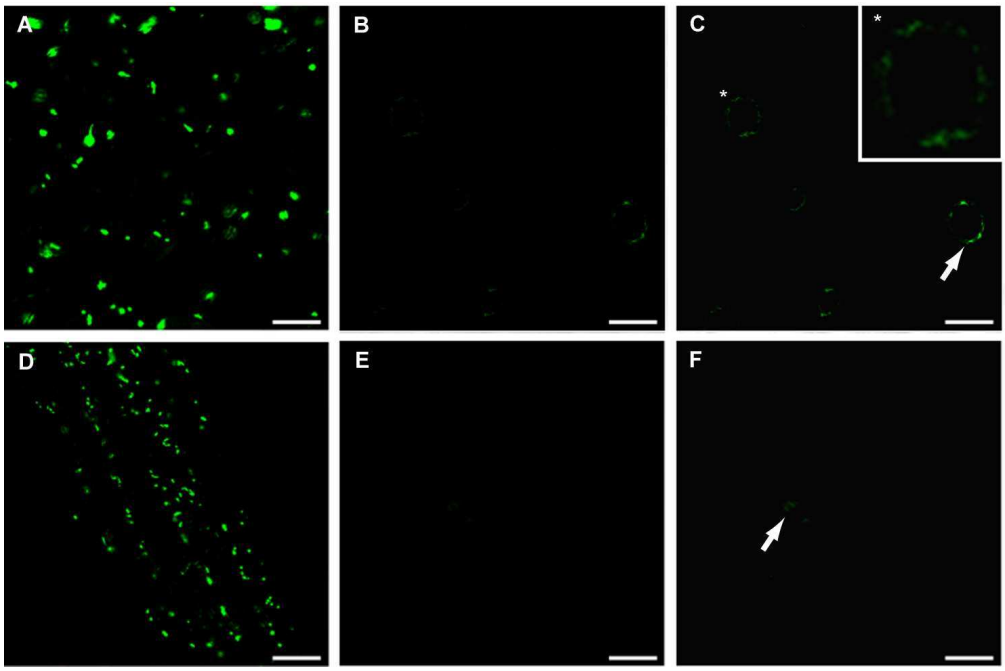


Seedlings transplanted onto 100 nM of LDS-003655 have low levels of PEX5, but PEX7 levels are unaffected
60x95mm (600 x 600 DPI)



Effect of 25 μM LDS-003655 on PTS1-mediated import in cotyledons and roots of Arabidopsis seedlings
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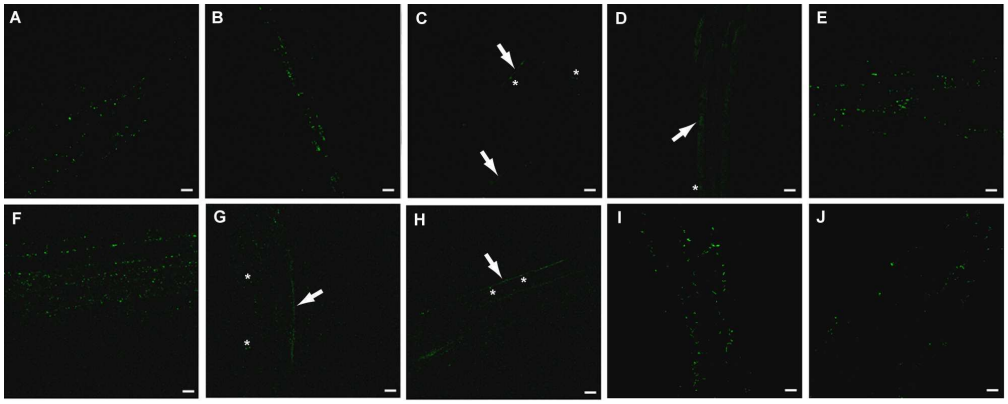

Effect of 25μM LDS-003655 analogues on PTS1-mediated protein import in roots of Arabidopsis seedlings
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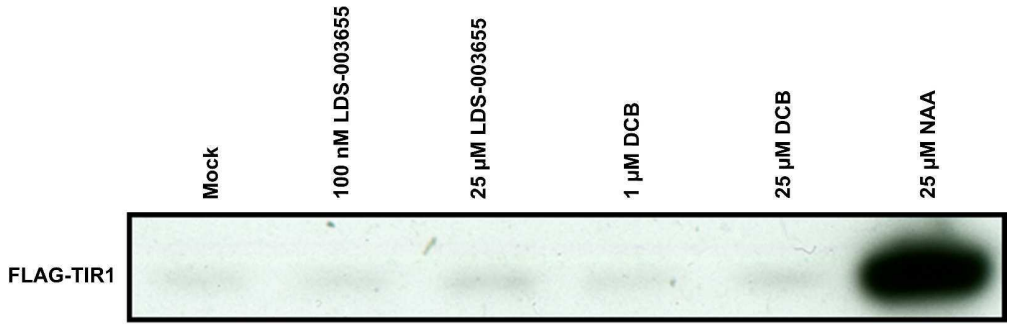

Unlike NAA, LDS-003655 and 2,5 dichlorobenzoic acid do not promote the interaction between TIR1 and IAA7
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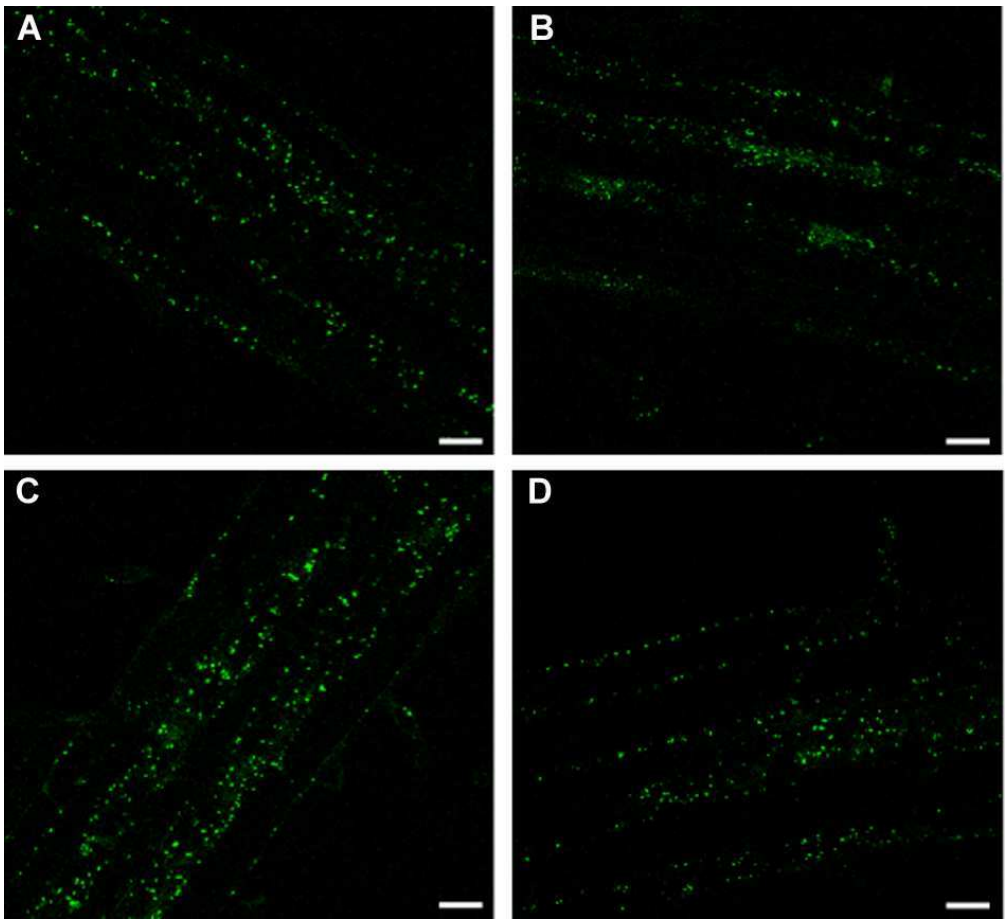

Import of CSY3-GFP, a PTS2 containing peroxisomal protein fused to GFP, does not appear to be significantly affected by LDS-003655 at 100 nM
80x72mm (300 x 300 DPI)