More Than Just Oligomannose: An N-glycomic Comparison of *Penicillium* Species*

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N-glycosylation is an essential set of post-translational modifications of proteins; in the case of filamentous fungi, N-glycans are present on a range of secreted and cell wall proteins. In this study, we have compared the glycans released by peptide/N-glycosidase F from proteolysed cell pellets of three *Penicillium* species (*P. dierckxii*, *P. nordicum* and *P. verrucosum* that all belong to the Eurotiomycetes). Although the major structures are all within the range Hex$_{5-11}$HexNAc$_2$ as shown by mass spectrometry, variations in reversed-phase chromatograms and MS/MS fragmentation patterns are indicative of differences in the actual structure. Hydrofluoric acid and mannosidase treatments revealed that the oligomannosidic glycans were not only in part modified with phosphoethanolamine residues and outer chain och1-dependent mannosylation, but that bisecting galactofuranose was present in a species-dependent manner. These data are the first to specifically show the modification of N-glycans in fungi with zwitterionic moieties. Furthermore, our results indicate that mere mass spectrometric screening is insufficient to reveal the subtly complex nature of N-glycosylation even within a single fungal genus. *Molecular & Cellular Proteomics* 15: 10.1074/mcp.M115.055061, 73–92, 2016.

Fungi and yeast have been used for hundreds of years as modifiers of foodstuffs, e.g. in fermentation, baking or cheese production. Ecologically significant in degradation of biological materials or as symbionts, they have within the last century also become biotechnologically important as sources of antibiotics, enzymes and recombinant proteins. On the other hand, their harmful potential is reflected by their ability to produce mycotoxins, their association with allergy or their pathogenicity toward plants and animals. For the fungal kingdom (1) currently five divisions are defined, with the Ascomycota and the Basidiomycota being the most familiar. Within the Ascomycota, the yeasts form a separate clade with unicellular species, whereas filamentous fungi including various molds are examples of multicellular mycelium-forming species. Among the most familiar of the latter is the class of Eurotiomycetes including *Penicillium* species (2), which are of general interest because of their production of antibiotics such as penicillin and griseofulvin as well as their role in food spoilage. Some species cause opportunistic infections as exemplified by *Penicillium* (Talaromyces) marneffei, which result in severe symptoms in immunocompromised humans and other animals (3). In terms of allergens, IgE reactivity toward Pen c 30 from *P. citrinum* is apparently reduced by periodate oxidation, suggesting IgE binding to the carboxydrate moieties of this protein (4).

The fungi and yeasts may be morphologically diverse, but share fundamental characteristics such as possessing a polysaccharide-rich cell wall. Furthermore, basic scientific research on the model ascomycete *Saccharomyces cerevisiae* has revealed conserved aspects of genetics and metabolism also in the field of glycoscience, e.g. by the study of the *alg* genes involved in the formation of N-glycans (5). Regarding the observed N- and O-glycans of fungi and yeasts hypermannosylation seems to be a general feature; here there is a key role for the Och1p $\alpha$1,6-mannosyltransferase which initiates the formation of ‘outer chains’ of variable lengths on the $\alpha$1,3-antennae. Additional modifications dependent on the species, with phosphodiesters, galactopyranose, N-acetylglucosamine or pyruvate residues, xylose and fucose have also been reported (6–8). In the case of some of the Eurotiomycetes, galactofuranose residues are also known to be present and glycan analyses of secreted glycoproteins have indicated that often one or more such residues can be present on the dominant Hex$_{5-10}$HexNAc$_2$ structures (9). Nevertheless, there are major gaps in our knowledge regarding the glycans and glycosylation pathways in most fungi. This is due, in part, to the fungal N-glycans containing primarily hexose residues, which presents a methodological challenge as compared with mammalian complex N-glycans with their variety of monosac-

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charide components. The *Penicillium* genus is exemplary for the lack of in-depth reports regarding the N-glycan structures of filamentous fungi in general.

As part of our general efforts directed at defining the fine structure of N-glycans from diverse species, we have compared the N-glycomes of three members of the *Penicillium* genus, including two mycotoxin producers. In addition to the typical high mannose glycans, in part featuring the precursors for ‘outer chain’ formation, we detected structures containing phosphoethanolamine and galactofuranose. The former modification is also known on glycoconjugates of, e.g. bacteria as well as of some eukaryotes such as trichomons and insects (10–12), whereas the latter is present not just in other fungi, but also in, e.g. mycobacteria and kinetoplasts (13, 14). Certainly, interactions of these glycan modifications with proteins of the human immune system are possible and may be relevant in terms of allergy or when considering these members of the Eurotiomycetes for manufacturing biotechnological products.

### EXPERIMENTAL PROCEDURES

#### Cultivation and Glycan Preparation

The *Penicillium* species were cultivated in ME broth medium (17 g malt extract and 3 g casein peptone per liter (Roth)). The collected mycelia (2–3 g wet weight) were first washed with 0.9% (w/v) NaCl and directly heat inactivated by boiling for 10 min prior to storage at −80 °C. After thawing, the material was lyophilized and then ground in liquid nitrogen. The powder was suspended in deionized water and acidified with formic acid to the final concentration of 5% (v/v). Porcine pepsin (2 mg; Sigma) was added for proteolysis overnight at 37 °C. The (glyco)peptides were purified according to our previously-published procedures (11). The N-glycans were subject to solid phase extraction on nonporous graphitized carbon (11). The N-glycans tides were purified according to our previously-published procedures (11). The N-glycans were then reconstituted in 0.1 M sodium acetate, pH 5, at 37 °C for 24 or 72 h; the specificities of the human immune system are possible and may be relevant in terms of allergy or when considering these members of the Eurotiomycetes for manufacturing biotechnological products.

#### N-glycan fractionation

Separation of PA-labeled glycans was carried out on a Shimadzu HPLC system equipped with a fluorescence detector (RF 20 AXS; excitation at 320 nm and emission at 400 nm). For reverse phase-high pressure liquid chromatography (RP-HPLC), a Hypersil ODS column (Agilent, Santa Clara, CA; a C18 column of the dimensions 250 × 4.6 mm) was used with 100 mM ammonium acetate, pH 4.0 (buffer A) and 30% (v/v) methanol (buffer B); after 5 min at 0% buffer B, a gradient of increasing buffer B (0.5% per minute) was programmed for 15 min. A pyridylaminated partial dextran hydrolysate was used for calibration in terms of glucose units; commercial Man9,GlcNAc2 (Takara, Shiga, Japan) and previously-defined Man6,GlcNAc2 A and B isomers were also chromatographed to verify elution times on the shallower gradient employed in this study. Selected RP-HPLC fractions were then subject to hydrophilic-interaction/anion-exchange (HIXA) using an IonPac AS11 column (Dionex, Sunnyvale, CA) as previously described (15). Buffer A was 0.8 M ammonium acetate, pH 3 and buffer B 80% acetonitrile. The following gradient was applied at a flow rate of 1 ml/min: 0–5 min, 99%; 5–50 min, 90%; 50–65 min, 80%; 65–85 min, 75% B. The HIXA column was calibrated using a mixture of oligomannosidic glycans (Man5,6,7,9GlcNAc3) derived from white beans.

**Mass Spectrometry**—Monoisotopic MALDI-TOF MS was performed using a Bruker Autoflex Speed (equipped with a 1000 Hz Smartbeam™-II laser) instrument in positive and negative reflectron modes with 6-aza-2-thiothymine (ATT) as matrix. MS/MS was performed by laser-induced dissociation (precursor ion selector was generally set to ±0.6%). The detector voltage was generally set at 1977 V for MS and 2133 V for MS/MS; 1000–3000 shots from different regions of the sample spots were summed. Spectra were processed with the manufacturer’s software (Bruker Flexanalysis 3.3.80) using the SNAP algorithm with a signal/noise threshold of 6 for MS (unsmoothed) and 3 for MS/MS (four-times smoothed). In total −3000 MS and MS/MS spectra were manually interpreted on the basis of the mass, fragmentation pattern and results of chemical and enzymatic treatments. The deviation between calculated and observed m/z values was typically 0.1–0.2 Da.

Selected Pyridylaminated N-glycans were also analyzed by online LC-MS/MS using a 10 cm × 150 μm i.d. column (5 μm porous graphitized carbon) coupled to an LTQ ion trap mass spectrometer (Thermo Scientific, Waltham, MA). Glycans were eluted using a linear gradient from 0–40% acetonitrile in 10 mM ammonium bicarbonate over 40 min at a flow rate of 10 μl/min and detected in negative-ion mode with an electrospray voltage of 3.5 kV, capillary voltage of −33.0 V and capillary temperature of 300 °C. MS/MS fragmentation was performed by collision induced dissociation with the collision energy set to 30% and air as sheath gas. The data were processed using the Xcalibur software (version 2.0.7, Thermo Scientific). Glycans were identified from their MS/MS spectra by manual annotation using the nomenclature of Domon and Costello (16).

Prior to GC-MS analyses of heptafluorobutyrate (HFB) derivatives (17), pyridylaminated oligosaccharides as well as standard monosaccharides were pyridylized in glass tubes with Teflon-lined screw caps and methanolysis was performed by addition of 200 μl 0.5 M methanolic HCl (80 °C for 20 h). The samples were then dried under a low stream of nitrogen and derivatized by the addition of 25 μl of heptafluorobutyric anhydride and 200 μl anhydrous acetonitrile (100 °C for 30 min). After cooling, the samples were again evaporated and dissolved in 50 μl of acetonitrile. HFB derivatives (1 μl) were injected onto a 7820A gas chromatograph (Agilent Technologies) equipped with a 30 m × 0.25 mm HP-5MS Ultra Inert capillary column (0.25 μm film phase; Agilent). The inlet temperature was 260 °C and the samples were then analyzed using the following temperature program: 100 °C for 1 min then 1.2 °C/min until 140 °C, followed by 4 °C/min from 140 °C to 240 °C and this temperature was maintained for 10 min. The GC apparatus was coupled to a 5975 MSD Series mass spectrometer (Agilent Technologies) with helium as carrier gas (1 ml/min). MS detection was achieved using electron impact ionization with single ion monitoring mode (m/z 169, 276, 277, 472, 551, 703). The temperature of the ion source was set at 230 °C and that of the quadrupole at 150 °C. The qualitative and quantitative analyses of the GC/MS data were obtained with the GC/MSD ChemStation software (Agilent Technologies).

**Mannosidase and Hydrofluoric Acid Treatments**—Further analysis by MALDI-TOF MS and MS/MS was performed after treatment with α-mannosidases (jack bean from Sigma, *Aspergillus* α1,2-specific from Prozyme, *Xanthomonas* mannosidase II or III, respectively α1,6- and α1,2/3- specific, from New England Biolabs, Ipswich, MA) in 25 mM ammonium acetate, pH 5, at 37 °C for 24 or 72 h; the specificities of the latter two mannosidases have been described with the α1,6-mannosidase only cleaving α1,6-mannose from otherwise unsubstituted residues (18, 19). For removal of galactofuranose or phosphoethanolamine residues, selected HPLC fractions were dried and incubated for 24 h at 0 °C with 3 μl 48% (v/v) hydrofluoric acid prior...
to evaporation; the samples were diluted in water and re-evaporated, before redissolving once again. Previous studies by us and others indicate that hydrofluoric acid removes phosphoesters (including phosphoethanolamine as well as methylphosphate, aminoethylphosphonates and phosphorylcholine), galactofuranose, and /H9251 1,2/3-fucose residues (11, 15, 20–23). In general, chemically or enzymatically treated glycans were reanalyzed by MALDI-TOF MS and MS/MS without further purification, but (where noted) samples were rechromatographed by RP-HPLC to determine the elution of the treated glycans in terms of glucose units.

RESULTS

Glycomic Screening of Penicillium Species—Initial screening of the pyridylminated N-glycomes of the peptic glycopeptides derived from P. dierckxii, P. nordicum and P. verrucosum was performed by MALDI-TOF MS in both positive and negative ion modes (Fig. 1 and Table I). The spectra in positive mode indicated the presence of a series of N-glycans with compositions in the range Hex4–14HexNAc2 ([M+H]+ in positive and [M-H]- in negative mode) and with abbreviated compositions of the form Hx14–12NPE0–3 (H, hexose; N, N-acetylhexosamine; PE, phosphoethanolamine). Further sodium and potassium adducts are not annotated; three peaks indicated with asterisks are contaminants removed by subsequent RP-HPLC. Intensity is shown in arbitrary units (a.u.).

HexNAc2 as compared with P. nordicum and P. verrucosum in which Hex9HexNAc2 or Hex10HexNAc2 were the major species. The positive mode spectra also showed some evidence for a modification of 123 Da on some glycans, especially in P. dierckxii. In negative ion mode, this modification was more pronounced in all three species with glycans of [M-H]- being observed; additionally, the Hex9–10HexNAc2 glycans in P. nordicum and P. verrucosum were also detected well in this mode.

Subsequently, the glycans were analyzed by RP-HPLC, a method first introduced for isomeric separation of pyridylminated glycans nearly thirty years ago (24, 25) and used by us for this purpose in a number of studies (15, 26), prior to MALDI-TOF MS analysis in both positive and negative mode of each fraction. A comparison of MALDI-TOF MS data for the glycomes and for individual HPLC fractions suggests a lack of in-source dissociation as the high (or low) intensity peaks detected by MS are indeed the most (or least) abundant as

FIG. 1. MALDI-TOF MS of the total N-glycan pools of three Penicillium species. Positive and negative ion mode spectra of pyridylaminated PNGase F-released N-glycans from P. dierckxii (A, D), P. nordicum (B, E) and P. verrucosum (C, F); major species are annotated with the m/z values ([M+H]+ in positive and [M-H]- in negative mode) and with abbreviated compositions of the form Hx14–12NPE0–3 (H, hexose; N, N-acetylhexosamine; PE, phosphoethanolamine). Further sodium and potassium adducts are not annotated; three peaks indicated with asterisks are contaminants removed by subsequent RP-HPLC. Intensity is shown in arbitrary units (a.u.).
Summary of N-glycan masses and compositions for three *Penicillium* species. Calculated m/z values for predicted N-glycans as [M+H]⁺ and [M-H]⁻ are listed; for observed values refer to the figures. All compositions are as HₓNᵧPE/PCz other than for the four marked with an asterisk, are corroborated with MS/MS data as well as, in many cases, by chemical and enzymatic treatments as discussed in the text. Qualitative assessments of abundance, based on fluorescence intensities of HPLC fractions, are indicated by +++, ++, +, trace or n.d. (not detected); H is hexose, N, N-acetylgalactosamine, PC phosphorylcholine and PE phosphoethanolamine.

| Composition | [M+H]⁺ | [M-H]⁻ | *P. nordicum* | *P. dierckxii* | *P. verrucosum* |
|-------------|--------|--------|---------------|---------------|-----------------|
| H4N2        | 1151.44| 1149.43| +             | +             | nd              |
| H5N2        | 1313.50| 1311.48| +             | +++           | +               |
| H5N2PE      | 1436.51| 1434.49| nd            | +             | nd              |
| H6N2        | 1475.55| 1473.54| +             | +++           | +               |
| H6N2PE      | 1598.56| 1596.55| trace         | +             | nd              |
| H7N2        | 1637.60| 1635.59| +             | +++           | +               |
| H7N2PC⁺     | 1640.61| 1638.59| trace         | nd            | nd              |
| H7N2PE      | 1760.61| 1758.60| trace         | +             | nd              |
| H8N2        | 1799.66| 1797.64| +++           | +++           | +               |
| H7N2PC⁺     | 1802.66| 1800.65| trace         | nd            | nd              |
| H7N2PE2     | 1883.62| 1881.61| nd            | +             | nd              |
| H8N2PE      | 1922.67| 1920.66| +             | +++           | nd              |
| H7N2PEPC⁺   | 1925.67| 1923.65| trace         | nd            | nd              |
| H9N2        | 1961.71| 1959.70| +++           | +++           | +               |
| H8N2PE2     | 2045.67| 2043.66| +             | +             | nd              |
| H9N2PE      | 2064.78| 2062.76| +++           | +++           | +               |
| H8N2PEPC⁺   | 2067.72| 2065.71| trace         | nd            | nd              |
| H10N2       | 2123.76| 2121.75| +             | +             | +++             |
| H9N2PE2     | 2207.73| 2205.71| trace         | +             | nd              |
| H10N2PE     | 2246.77| 2244.76| +             | +++           | +               |
| H11N2       | 2285.82| 2283.80| +++           | trace         | ++              |
| H10N2PE2    | 2369.78| 2367.77| nd            | +             | nd              |
| H11N2PE     | 2408.82| 2406.81| +++           | +             | ++              |
| H12N2       | 2447.87| 2445.85| +             | trace         | ++              |
| H10N2PE3    | 2492.79| 2490.77| nd            | +             | nd              |
| H11N2PE2    | 2531.83| 2529.82| +             | trace         | nd              |
| H12N2PE     | 2570.88| 2568.86| trace         | trace         | ++              |
| H13N2       | 2609.92| 2607.91| +             | trace         | +               |
| H13N2PE     | 2732.93| 2730.92| trace         | trace         | +               |
| H14N2       | 2771.97| 2769.96| trace         | nd            | +               |
| H14N2PE     | 2894.98| 2892.97| trace         | nd            | nd              |
| H15N2       | 2934.03| 2932.01| trace         | nd            | trace           |
| H16N2       | 3096.08| 3094.06| trace         | nd            | trace           |

The identification of isomers of oligomannosidic glycans in this study is based on RP-HPLC elution properties, MS/MS and α-mannosidase digests. The relative elution times of N-glycan biosynthetic intermediates (Fig. 2M) were comparable with literature values for RP-HPLC of oligomannosidic glycans (27), although the absolute glucose units slightly varied because of the somewhat flattened gradient employed (0.5% per minutes as compared with 1% per minute); also, in order to test the separation under these conditions, commercial Man₁₀GlcNAc₂ and previously-defined Man₁₂GlcNAc₂ isomers were run (supplemental Fig. S1). Generally, based on our observations from these and other species, the most intense fragments for oligomannosidic structures are Y3β ions corresponding to loss of the α₁,6-antennae (B and C branches), while retaining the α₁,3-arm (A branch; for a definition of the branches and example spectra, see Fig. 2). Furthermore, assignments of oligomannosidic glycans, e.g. Man₁₂GlcNAc₂,
can be made on the basis of loss of three or four residues with a specific α1,2-mannosidase (down to m/z 1313) or of seven or eight residues when using jack bean α-mannosidase (product of m/z 665; supplemental Fig. S2). Longer fully-mannosidase sensitive structures with larger Y3β fragments (e.g. m/z 1475) were concluded to be products of the Och1p α1,6-mannosyltransferase known to initiate lower arm “outer chain” formation in many fungal species (28). On the other hand, a number of partly mannosidase-resistant glycans displayed hydrofluoric acid sensitivity compatible with the presence of phosphoethanolamine and/or galactofuranose residues, which have been previously reported as components of fungal cell walls (29).

The N-glycome of P. nordicum—The simplest chromatogram of the three glycomes was probably that of P. nordicum, which also displayed the least glycan heterogeneity in individual fractions (Fig. 3). Considering the individual fractions, the major structure had the composition Hex₆HexNAc₂ co-eluting with standard Man⁶GlcNAc₂, which is also the major glycan detected by MALDI-TOF MS of the overall glycome (Fig. 1B); furthermore, as particularly visible in negative mode, a portion of the Hexᵢ₋₀HexNAcᵢ griefs was decorated with one or two moieties of 123 Da (Fig. 1E).

The identification of typical Man₅₋₉GlcNAc₂ isoforms was verified by the MS/MS fragmentation before and after digestion with α-mannosidases. As introduced above, MS/MS of a “Golgi-processed” Man₆GlcNAc₂ (6.7 g.u.) and the form of Man₇GlcNAc₂ (7.2 g.u.) still possessing the ‘middle’ α1,2-mannose residue (B mannosyl) showed m/z 827 Y-fragments which are interpreted as resulting from the loss of the entire α1,6-antenna (Fig. 2 J and 2K); the two Man₉GlcNAc₂ isomers (A and C; 5.3 and 6.2 g.u.) as well as the Man₇GlcNAc₂ (6.4 g.u.) and Man₈GlcNAc₂ (5.0 g.u.) displayed upon MS/MS either m/z 989 or 1151 typical Y-fragments reflecting different lengths of the α1,3-antenna (“A” branch; see Fig. 2 A, 2C, 2D, and 2G). Furthermore, based on its elution position and fragmentation (Fig. 2L), a Hex₆HexNAc₂ structure, which only lost maximally four residues upon jack bean α-mannosidase digestion, was concluded to be a glucosylated variant of Man8C.
For the Hex$_{11}$HexNAc$_2$ glycan (the second most abundant glycan; 4.6 g.u.), sequential digestion with $\alpha_{1,2}$- and $\alpha_{1,6}$-mannosidase in conjunction with MS/MS, indicated that this glycan corresponded to a standard Man$_9$GlcNAc$_2$ glycan modified with two extra mannose residues on the $\alpha_{1,3}$-antenna (Fig. 4). Specifically, the initial MS/MS of the $m/z$ 2285 species showed a dominant $m/z$ 1475 fragment (Fig. 4A); removal of five mannose residues by $\alpha_{1,2}$-mannosidase resulted in an $m/z$ 1475 product with a major $m/z$ 989 fragment (Fig. 4B). Subsequent $\alpha_{1,6}$-mannosidase treatment resulted in the loss of one mannose and in a major $m/z$ 827 fragment when analyzed by MS/MS (Fig. 4C); these data are consistent with the presence of an outer chain Man$_{\alpha,1,6}$ residue directly attached to the core $\alpha_{1,3}$-mannose.

A similar approach was used to examine a minor later-eluting Hex$_9$HexNAc$_2$ present in the same fraction as Man$_8$A (5.3 g.u.; Fig. 4D). The fragmentation pattern of this Hex$_{\alpha}$- HexNAc$_2$ isomer was similar to the standard Man$_9$GlcNAc$_2$; however, only three mannose residues were released by $\alpha_{1,2}$-mannosidase resulting in $m/z$ 1475 with a $m/z$ 989 fragment (Fig. 4E), in contrast to removal of four in the case of standard Man$_9$GlcNAc$_2$ (supplemental Fig. S2). Thereafter, $\alpha_{1,6}$-man-
nosidase could also release one mannose from the α1,3-antenna, whereas the nonreducing α1,6-mannose on the “upper” antenna was resistant to this treatment (Fig. 4F; also as judged by the MS/MS data showing a dominant m/z 827 fragment). Therefore we conclude that the minor Hex₆₋HexNAC₂ isomer is an Man8A-type glycan modified by Och1, the α1,6-mannosyltransferase which initiates fungal/yeast-type outer chain formation (28). Indeed, the requirement to first treat Och1-modified oligosaccharides with α1,2-mannosidase before the α1,6-mannose can be removed was previously shown for a bona fide product of recombinant Och1 mannosyltransferase (30). However, in accordance to the literature, (19) the α1,6-mannosidase does not remove the nonreducing C-branch α1,6-mannose from “Golgi-type” Man₉GlcNAc₂ because of it being attached to a disubstituted mannose (see also Scheme in Fig. 4). Thus both exoglycosidase sensitivity and elution time can distinguish the standard and Och1-product isomers of Man₉GlcNAc₂.

We then examined the set of glycans with the modification of 123 Da; MS/MS analysis showed the presence of key B- and Y-fragments such as m/z 448 (Hex₆PE₁), 934 (Hex₆PE₁), 1112 (Hex₇HexNAC₂PE₁-PA), 1274 (Hex₇HexNAC₂PE₁-PA), and 1598 (Hex₆HexNAC₂PE₁-PA) in positive mode or m/z 1055 (Hex₆PE₁) in negative mode (see Fig. 5 for example MS/MS data). As the mass difference and the strong signal in negative mode MS was reminiscent of properties of phosphoethanolamine-modified glycans from Trichomonas vaginalis (11), we treated such glycans with hydrofluoric acid, which is known to cleave phosphodiester bonds (21). Indeed, both the 123 Da modification and the strong ionizability in negative mode were lost when such treatment was performed on these glycans (see below).

The next question was to localize the residue modified by phosphoethanolamine (PE); by examining the MS and MS/MS data upon mannosidase digestions before and after hydrofluoric acid treatment we show that the sub-terminal C-branch mannose on the α1,6-antenna was the major location for modification by phosphoethanolamine, regardless of whether the basic structure was Man₉GlcNAc₂ (Man8A), Man₉GlcNAc₂, or Man₁, GlcNAc₂ (Fig. 5A–5C; Y-fragments of m/z 989, 1151, and 1475 being indicative of an unmodified A-branch, whereas the m/z 934 ion, Hex₆PE₁, was consistent with a phosphoethanolamine modification on the C branch). For the putative Man₉GlcNAc₂PE₁ glycan (m/z 2084; 4.0 g.u.), initially, hydrofluoric acid treatment resulted in the loss of 123 Da (compare Fig. 6A and 6B) and co-elution by RP-HPLC with the nonmodified form of Man₉GlcNAc₂ (5.0 g.u.; supplemental Fig. S3). For the sequential digestion, the glycan was first treated with α1,2-mannosidase, which resulted in the loss of four mannose residues (Fig. 6C); subsequent treatment with the “nonspecific” jack bean α-mannosidase yielded a product of m/z 1112 (putatively Man₉GlcNAc₂PE₁; Fig. 6D). Only after hydrofluoric acid treatment could α1,6-mannosidase release the final two α-mannose residues (Fig. 6E and 6F), which is consistent with the subterminal phosphoethanolamine modification “blocking” the second α1,6-linked mannose on the α1,6-antenna. A similar approach was performed on Man₁, GlcNAc₂PE₁ (3.3 g.u.; Fig. 5C) and the results allowed us to conclude the same location of the phosphoethanolamine modification; analogous to the aforementioned experiments with Man₁, GlcNAc₂, the Och1 modification could also be proven by the α1,6-mannosidase sensitivity.

In the case of three glycans carrying two phosphoethanolamine residues (Man₉GlcNAc₂PE₁ and Man₁, GlcNAc₂PE₂), MS/MS before (Fig. 5D, 5F, and 5H) and after α1,2-mannosidase digestion (Fig. 5E, 5G, and 5I) indicated that the subterminal mannose on the α1,3-antenna (A-branch) was a further location for this modification; specifically, this was shown by the shift of the key fragments from m/z 1112, 1274, and 1598 respectively to m/z 950, 1112, and 1274. Additionally, a minor Man₁, GlcNAc₂PE₂ isomer had two phosphoethanolamine residues on the α1,6-antenna as indicated by the m/z 1055 fragment (Hex₆PE₂ in negative mode; see Fig. 5J). Furthermore, minor amounts of glycans with phosphorylcholine were present in fractions >8 g.u.; diagnostic for this modification are dominant B-fragments of m/z 328 and 490 (Hex₆PC₁; see Fig. 5K for MS/MS of Hex₆HexNAC₂PC₁). Because of the low amounts, the phosphorylcholine-modified glycans were not further analyzed, whereas only compositions can be predicted for glycans carrying both zwitterionic moieties (Hex₇/εHexNAC₂PE₁PC₁; see Table I).

The N-glycome of P. dierckxii—As mentioned above, the major N-glycans observed for P. dierckxii were more variable in terms of size than for the other two glycomes and a higher proportion of the N-glycans had the composition Hex₆/εHexNAC₂PC₀₋₂ (Fig. 1A). In general, the number of RP-HPLC fractions is higher and the retention time tangentially shifted to lower glucose unit values than for the other two species studied (Fig. 7). MS of the individual fractions revealed that the early fractions (<4 g.u.) contain only glycans modified with phosphoethanolamine; the glycans with multiple such modifications were particularly poorly retained on the column. The standard oligomannosidic glycans eluted in the range 4.8–6.8 g.u.; however, this region also contained some phosphoethanolamine-modified glycans, whereby such glycans eluted ~1–2 g.u. earlier than the corresponding “parent” neutral form.

Among the neutral oligomannosidic glycans, the major structure was concluded to be Man8B; this is supported by the retention time (4.7 g.u.; Fig. 7) and MS/MS data (key Y-fragment of m/z 1151; Fig. 2B). Of the other neutral glycans, retention times and fragmentation patterns indicate that the dominant forms of Man₉GlcNAc₂ lack the middle “B” mannose (see Fig. 2E, 2F, 2H, and 2I). This is unlike the case in P. nordicum in which the Man₉GlcNAc₂ is the late-eluting form retaining the B-mannose (see above). The last eluting HPLC fraction contains solely the standard Golgi-processed form of Man₉GlcNAc₂; its abundance in this species is also obvious.
Fig. 5. Analysis of the major phosphoethanolamine-modified glycans from *P. nordicum*. MALDI-TOF MS/MS spectra of (A, D) Man₈GlcNAc₂PE₁₋₂, (B, F) Man₉GlcNAc₂PE₁₋₂, (C) Man₁₁GlcNAc₂PE₁, two forms of (H, J) Man₁₁GlcNAc₂PE₂ and (K) one phosphorylcholine-modified glycan Hex₆HexNAc₂PC₁. The MS/MS were recorded in positive mode except for MS/MS of m/z 2529 in negative mode (J); the positions of the phosphoethanolamine (PE) modifications are inferred from mannosidase digestion data (see Fig. 6). Man₈,₉,₁₁GlcNAc₂PE₂ were subject to α₁,₂-mannosidase treatment in order to prove the sub-terminal location of the phosphoethanolamine residues (E, G, I). The dominant fragments are of the Y-series which contain the GlcNAc-PA, whereas the detected B-fragments contain the zwitterionic phosphoethanolamine or phosphorylcholine residues.
from the MALDI-TOF MS profile. Och1-modified glycans (Man\textsubscript{9}/\textsubscript{10}GlcNAc\textsubscript{2}) were also found; unlike the Och1-modified Man\textsubscript{9}GlcNAc\textsubscript{2} from \textit{P. nordicum} with a short A-branch, the isoform from \textit{P. dierckxii} lacks a B-branch mannose (for MS/MS see supplemental Fig. S4). Further corroborative evidence for the proposed oligomannosidic structures as well as the Och1-modification is derived from \textit{H.9251} mannosidase digests (supplemental Figs. S2 and S4).

The negative mode complete spectrum (Fig. 1D) indicated that the dominant glycans with the 123 Da modification were of the predicted form Hex\textsubscript{8–9}HexNAc\textsubscript{2}PE\textsubscript{1}; overall, when considering the MS and MS/MS data on individual RP-HPLC fractions, the putatively phosphoethanolamine-modified N-glycans ranged from Hex\textsubscript{5–10}HexNAc\textsubscript{2}PE\textsubscript{1–3} (for example MS/MS data, see Fig. 8). Based on the MS data and the intensity of fluorescence, we also conclude that such glycans constitute perhaps half of the entire glycome. The locations of the phosphoethanolamine modifications were determined, similarly as for \textit{P. nordicum}, by a combination of mannosidase and hydrofluoric acid treatments and interpretation of MS/MS data. In general, in the case of a single phosphoethanolamine moiety, neutral Y-fragments of \textit{m/z} 989, 1151, 1313, and 1475 and zwitterionic B-fragments of \textit{m/z} 448 and 772 were compatible with modification of the C-antenna and with absence of a mannose from the B-arm (Fig. 8C–8G). Exceptions to this were Hex\textsubscript{5–6}HexNAc\textsubscript{2}PE\textsubscript{1} isomers with fragments of \textit{m/z} 950 and 1112 indicative of modification of the A-branch (Fig. 8A and 8B); further lower “A-branch” Y3\beta-fragnents (\textit{m/z} 1112, 1274, and 1436) were also observed for the doubly-modified glycans (Fig. 8I–8K). Because of the higher amount, the triply-modified glycan (Hex\textsubscript{8–9}HexNAc\textsubscript{2}PE\textsubscript{3}) was analyzable in positive mode and an \textit{m/z} 1057 fragment (Hex\textsubscript{3}PE\textsubscript{3}) provided evidence for substitution of both the B and C branches (Fig. 8L).

In this species, the structure of these glycans differed as compared with those of the same composition in \textit{P. nordicum}; this is because of the retention of the A and C mannoses and frequent loss of the B-mannose, which pertains for the neutral

Fig. 6. Enzymatic and chemical treatment of the Man\textsubscript{9}GlcNAc\textsubscript{2}PE\textsubscript{1} glycan from \textit{P. nordicum}. MALDI-TOF MS analysis of the glycan before (A) or after (B) hydrofluoric acid treatment as well as of the glycan treated sequentially with (C) α1,2-mannosidase, (D) “nonspecific” jack bean α-mannosidase, (E) hydrofluoric acid, and finally (F) α1,6-mannosidase. Key fragments (putatively Y3\beta) in MS/MS are given in parentheses. For MS/MS of the untreated glycan, see Fig. 5B; the effect of hydrofluoric acid on the RP-HPLC retention time (shift from 4 to 5 g.u.) is shown in supplemental Fig. S3. The \textit{m/z} 665 in panel D originates from jack bean mannosidase digestion of a series of co-eluting low abundance unmodified oligomannosidic glycans in the original fraction (not annotated), but because of efficient ionization this species is overrepresented in terms of signal height.
FIG. 7. RP-HPLC analysis of pyridylaminated N-glycans of *Penicillium dierckxii*. The major fractions in the chromatogram are annotated with structures proposed on the basis of elution, MS/MS and digestion data. Depictions are according to the nomenclature of the Consortium for Functional Glycomics (circles, mannose; squares, N-acetylglucosamine; PE, phosphoethanolamine). Note that, in comparison to the other two species, the Man₅GlcNAc₂ structure (6.7 g.u) is rather dominant, which is consistent with the overall MALDI-TOF MS profile (see Fig. 1A).

FIG. 8. MS/MS analysis of phosphoethanolamine-modified N-glycans from *P. dierckxii*. MALDI-TOF MS/MS spectra of (A) Hex₅HexNAc₂PE₁, (B, C) two forms of Hex₆HexNAc₂PE₁, (D–G) Hex₇–₉HexNAc₂PE₁, (G–H) two forms of Hex₁₀HexNAc₂PE₁, (I–K) Hex₇–₉HexNAc₂PE₂, and (L) Hex₁₀HexNAc₂PE₃ are annotated with key fragments. The B-fragments of m/z 772 (Hex₄PE₁; D–G) correlate with the lack of one mannose from the B-branch, whereas the m/z 934 ions (Hex₅PE₁) are akin to those found in the other two species. The presence of dominant putative Y₃/Y₆ fragments of m/z 1436 and 1721 (J, L) in which the A branch is lost, in contrast to the generally-observed loss of the B/C branches, is probably because of the higher ionization potential associated with the phosphoethanolamine.
and the zwitterionic glycans and also results in differences in elution time (i.e. Man$_8$GlcNAc$_2$PE$_{1–2}$ glycans from *P. dierckxii* are less retained than the corresponding *P. nordicum* isomers, which correlates with the different degree of retention of the respective dominant parent Man$_8$B and Man$_8$A backbone structures; compare Figs. 3 and 7). Furthermore, the lack of the B-mannose affects the position and number of phosphoethanolamine residues on the mannosidase-processed glycans; also modifications of both the A and C-branches were observed in the case of Hex$_5/6$HexNAc$_2$PE$_1$ with the C-isomers eluting earlier than the A-isomers (see annotations on Fig. 7). Most frequent were subterminal positions for the zwitterionic modification, but terminal positions on some Man$_5$–6GlcNAc$_2$PE$_1$ isomers were also found. Outer chain modifications were also present in this species with forms of Hex$_8$–10HexNAc$_2$PE$_{1–3}$ concluded to possess a Man$_1$,6- modification of the α1,3-antenna (Fig. 8L) and of two digestion products in the insets).

An example for our further analyses is shown for Hex$_7$HexNAc$_2$PE$_2$ (m/z 1883; major Y-fragment of 1112; Fig. 8I). Sequential treatment (Fig. 9) with α1,2-mannosidase and jack bean α-mannosidase resulted in products of m/z 1559 (Hex$_5$HexNAc$_2$PE$_2$) and 1397 (Hex$_4$HexNAc$_2$PE$_2$; both with major fragments of m/z 950). Subsequently, the serial treatments with hydrofluoric acid, α1,2/3-mannosidase and α1,6-mannosidase yielded respectively m/z 1151 (Hex$_4$HexNAc$_2$), 989 (Hex$_3$HexNAc$_2$) and 665 (Hex$_1$HexNAc$_2$). The conclusion is, as for the dimodified Man$_8$A from *P. nordicum*, that phosphoethanolamine modifies the second α1,6-mannose on the α1,6-antenna and the subterminal core α1,3-mannose. A simplified form of this procedure was also performed with Hex$_8$HexNAc$_2$PE$_1$, which allowed us to verify the modification of the α1,6-antenna of a Man$_8$B isomer (supplemental Fig. S5).

The N-glycome of *P. verrucosum*—In terms of the overall spectra, MALDI-TOF MS suggested that the N-glycome of *P.
verrucosum (Fig. 1C and 1F) is rather similar to that of *P. nordicum*. Surprisingly the HPLC chromatogram is quite different. The major bulk of the glycans of *P. verrucosum* eluted in the range of 5–6 g.u. (Fig. 10) and these fractions contained a complex mixture of glycans; the multiplicity of glycans in two regions of the RP-HPLC chromatogram led us to perform HIAAX on two sub-pools (3.9–4.3 g.u. and 5.2–6.0 g.u.). Particularly striking were the multiple forms of Hex8–11HexNAc2 and the relatively late RP-HPLC retention times of many N-glycans. We wondered whether this was because of the presence of an unusual hexose residue and, as galactofuranose is known in *Aspergillus* and previously shown to be sensitive to hydrofluoric acid (20), treatment with this reagent was performed on a number of fractions as well as on the entire pool. The resulting shift in the N-glycome was shown by loss of one hexose from some glycans and loss of the late-eluting RP-HPLC fractions (supplemental Fig. S6). On the other hand, some earlier-eluting glycans were also sensitive to hydrofluoric acid as shown by losses of 123 (one phosphoethanolamine) and 285 (one phosphoethanolamine and one hexose; see below).

The glycans insensitive to hydrofluoric acid were primarily eluting around 5 g.u. (Hex8–11HexNAc2) and 7 g.u. (Hex5–7HexNAc2). Based on the retention time (also in comparison to the other species), MS/MS fragmentation and /H9251,1,2-mannosidase sensitivity, the fractions around 5 g.u. were con-

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**Fig. 10.** RP-HPLC analysis of pyridylaminated N-glycans of *Penicillium verrucosum*. The major fractions in the chromatogram are annotated with structures proposed on the basis of elution, MS/MS and digestion data; the lower panels display the HIAAX chromatograms of the two indicated RP-HPLC sub-pools. The analysis of the Hex8HexNAc2 structures labeled A and B are shown in Fig. 11, whereas the HIAAX fractions annotated with roman I and II correspond to the two isobaric HexHexNAcPE structures whose digestion patterns are shown in supplemental Fig. S8.
incuded to contain Man8B (4.7 g.u.), a standard Man9GlcNAc2 (5.0 g.u.) and a Man11GlcNAc2 with a Man1,2Man1,6-modification; there were also putative Man9–10GlcNAc2 isomers with a single “outer chain” α,1,6-mannose. The Man5GlcNAc2 was a standard “Golgi” isomer (6.7 g.u.; major Y-fragment m/z 827) and the Man7GlcNAc2 apparently contained the B mannos (6.4 g.u.; major fragment m/z 989) as in the case with P. nordicum (Fig. 2G).

Because of their nonstandard nature, a particular focus was on the later-eluting isomers of the Hex6–11HexNAc2 glycans; GC-MS of the untreated 5.5–6.5 g.u. region indicated a 1.4:9.3:1 ratio of galactose/mannose/GlcNAc as opposed to 0.3:9.0:1 in the RP-HPLC pool after hydrofluoric acid treatment (supplemental Fig. S6). As the reducing-terminal GlcNAc-PA cannot be detected as an HFB derivative, the monosaccharide ratio for the glycans sensitive to hydrofluoric acid supports the presence of maximally one galactofuranose per glycan.

In terms of individual glycans, an example is the analysis of the Hex10HexNAc2 (m/z 2123; Fig. 12A) eluting at 5.5 g.u., which is in the region subsequently subject to HIAx fractionation in order to isolate single glycans; MS/MS data shows a key m/z 1313 Y-fragment suggestive of loss of the upper α,1,6-antenna (Fig. 12B). Treatment with hydrofluoric acid resulted in loss of one hexose (concluded to be galactofuranose; Fig. 11B and 12B) which was accompanied by a change in the main fragment to m/z 1151, whereas digestion with α,1,2-mannosidase resulted in loss of four hexose residues (Fig. 11C) and subsequently jack bean mannosidase resulted in loss of another two hexose residues resulting in a Hex4HexNAc2 structure (Fig. 11D and 12H); hydrofluoric acid treatment resulted in loss of a further hexose (Fig. 11E) and

Fig. 11. Enzymatic and chemical treatment of isobaric forms of Hex10HexNAc2 from P. verrucosum. MALDI-TOF MS analysis of the glycan of Hex10HexNAc2 eluting at 5.5 g.u. (isomer A) and subsequently further purified by HIAx before (A) or after treatment with hydrofluoric acid (B) or a sequential treatment with α,1,2-mannosidase (C), jack bean mannosidase (D), hydrofluoric acid (E), and finally α,1,2/3-mannosidase (F). Similarly, the results of MALDI-TOF MS of a second form of Hex10HexNAc2 (6.0 g.u.; isomer B) before (G) or after sequential digestion with hydrofluoric acid (H), α,1,2-mannosidase (I) and finally α,1,6-mannosidase (J). The Hex6HexNAc2 and Hex7HexNAc2 digestion products shown in panels B and E were verified by RP-HPLC to co-elute with authentic Man9GlcNAc2 and Man9GlcNAc2 standards (see supplemental Fig. S3). The MS/MS before and after hydrofluoric acid treatment of isomer A is shown in Fig. 12B.
finally α1,2/3-mannosidase removed another residue to result in Hex9HexNAc2 (Fig. 11F). Hydrofluoric acid treatment of the original Hex9HexNAc2 and of the jack bean mannosidase digested glycan resulted in respective co-elutions with standard Man9GlcNAc2 (5.0 g.u.; major fragment m/z 1151) and Man9GlcNAc2 (supplemental Fig. S3); also, forms of Hex10HexNAc2 and Hex11HexNAc2 (for MS/MS, see Fig. 12A and 12C) were also sensitive to hydrofluoric acid with a corresponding alteration in the m/z of the major fragment (supplemental Fig. S7).
The sum of these data suggested that the galactofuranose residue is rather "deep" within the structure, particularly as the galactofuranose does not block removal of peripheral residues during mannosidase digestion. There are a number of models for potential "residual" Gal\textsubscript{1,6}Man\textsubscript{3}GlcNAc\textsubscript{2} structures; based also on ruling out some theoretical MS1/MS and digestion outcomes as well as showing that the hydrofluoric acid Man\textsubscript{3}GlcNAc\textsubscript{2} product co-elutes with authentic Man\textsubscript{3}GlcNAc\textsubscript{2}, we postulate a bisecting position for this residue. This position sterically hinders removal of the α,1,3- and α,1,6-mannose residues of Man\textsubscript{4}GlcNAc\textsubscript{2}, as observed for the jack bean mannosidase digest. An overall composition of Gal\textsubscript{1,6}-Man\textsubscript{3}GlcNAc\textsubscript{2} is thereby proposed for the Hex\textsubscript{10}HexNAc\textsubscript{2} structure eluting at 5.5 g.u. Similarly, a combination of hydrofluoric acid, α1,2-mannosidase and α1,6-mannosidase was used to reveal that a second form of Hex\textsubscript{10}HexNAc\textsubscript{2} (6.0 g.u.) had a different structure with a single "outer chain" α1,6-mannose, but also carried galactofuranose (Fig. 11G–11J).

Another indication for a bisected glycan comes from a negative ion mode LC-MS\textsuperscript{n} experiment on a jack bean mannosidase digest, yielding Hex\textsubscript{10}HexNAc\textsubscript{2}−PA (m/z 1149), which resulted in observation of cross-ring cleavages indicative for tri-substitution of the β-mannose. Specifically, the lack of ions at m/z 323 (mass of two hexoses in series) and at m/z 413 (0,2A fragment of β-Man) suggests that the β-Man is substituted with three hexose residues, whereas the ion at m/z 443 is because of cross-ring cleavage of the β-Man (0,2A-Hex; Fig. 12H). Although a 0,2A ion (m/z 605) was not observed (which is specific for a C4-substitution), the mannosidase resistance of the galactofuranose-modified core region is comparable with that of bisected N-glycans (3,4,6-substitution of the β-mannose) as opposed to the ability of mannosidasises to remove α-mannose residues attached to a 2,3,6-substituted β-mannose as found in β2-xylosylated plant oligosaccharides.

The set of glycans putatively carrying phosphoethanolamine and the galactofuranose eluted in the region 3.3–4.5 g.u. (Hex\textsubscript{9}–12HexNAc\textsubscript{2}PE\textsubscript{1}); the fractions in the region 3.9–4.3 g.u. were also, as a form of 2D-HPLC, refractionated by HiAX in order to resolve the glycans further according to size. The two forms of Hex\textsubscript{9}HexNAc\textsubscript{2}PE\textsubscript{1} eluting in two different HiAX fractions (supplemental Fig. S8A and S8D) showed MS/MS Y-fragments of m/z 1151 (Fig. 12D and 12E); because of the different elution, we assumed that these were isobaric structures and, therefore, subjected both to hydrofluoric acid treatment (supplemental Fig. S8B and S8E). The slightly-later eluting structure (isobar I) lost a single phosphoethanolamine, whereas the earlier one (isobar II) additionally lost a hexose (putatively galactofuranose); subsequent α1,2-mannosidase treatment resulted in both yielding products of m/z 1313 (supplemental Fig. S8C and S8F). The conclusion was that isobar I was based on a standard Man\textsubscript{4}GlcNAc\textsubscript{2}, as found to elute at 4 g.u. in the case of the other two Penicillium species (see MS/MS in Fig. 5B), and isobar II on Gal\textsubscript{1,6}Man\textsubscript{3}GlcNAc\textsubscript{2}. Hydrofluoric acid treatment of another 2D-fractionated glycan (Hex\textsubscript{10}HexNAc\textsubscript{2}−PE\textsubscript{1}) also resulted in loss of one hexose and one phosphoethanolamine (supplemental Fig. S8G–S8J). Furthermore, MS/MS of Hex\textsubscript{10}HexNAc\textsubscript{2}PE\textsubscript{1} (Fig. 12F and 12G) resulted in dominant m/z 1313 or 1476 Y-fragments (Hex\textsubscript{5}−HexNAc\textsubscript{2}PA) as well as B-fragments of m/z 448, 610, 772, and 934 (Hex\textsubscript{5}−DEI); these data are again compatible with the presence of the phosphoethanolamine on the α1,6-antenna and a bisecting galactofuranose. Neither glycans with two phosphoethanolamine modifications nor fragments indicating phosphoethanolamine on the A branch were observed in this species.

**DISCUSSION**

*More Than Just Mannose: The N-glycomes of Eurotiomycetes—As for many fungi, the Penicillium genus is relatively poorly studied in terms of exact glycan structures. Previous reports on the N-glycans of Penicillium or other members of the Eurotiomycetes such as Aspergillus species have been generally centered on secreted enzymes, especially various hydrodases, with either mass spectrometric or electrophoretic methods being employed. Examples of the data on single glycoproteins include *Aspergillus oryzae* amylase (Man\textsubscript{9}−6GlcNAc\textsubscript{2}) (31), *A. niger* α-galactosidase (Hex\textsubscript{7}−26−HexNAc\textsubscript{2}) (32), *P. minioluteum* dextranase (Hex\textsubscript{5}−9HexNAc\textsubscript{2}) (33), *Penicillium sp.* β-galactosidase (Hex\textsubscript{5}−7HexNAc\textsubscript{2}) (34), *P. canescens* α-arabinofuranosidase (up to Hex\textsubscript{2}HexNAc\textsubscript{2}) (35), and *P. notatum* (P. chrysogenum) phospholipase (Man\textsubscript{9}−GlcNAc\textsubscript{2}) (36). Only in a few cases were the types and linkages of the hexose residues defined.

In contrast we define not only the type of hexose, but also often the linkage within the N-glycan as well as demonstrate for the first time the presence of zwitterionic modifications of N-glycans of fungal species. We have examined cellular glycoproteins, whose side chains are not cross-linked to the cell wall of these species, and have employed the approach of ‘off-line’ LC-MALDI-TOF-MS analysis, in conjunction with chemical and enzymatic treatments, of fluorescently-labeled N-glycans in their native state. The range of basic structures (Hex\textsubscript{5}−1HexNAc\textsubscript{2}) may be within that of many previous studies; however, as shown by our comparison of *P. nordicum* and *P. verrucosum*, which have rather similar overall MALDI-TOF mass spectral profiles, the use of mass spectrometry to determine composition alone is misleading. A necessary requirement for glycomic analyses, even of fungi, is the in-depth examination of the individual fractions, which shows that glycans of the same mass have quite different structures. Thus, separation by RP-HPLC reveals a more complex picture than the use of mass spectrometry or electrophoresis alone and offers isomeric separation of oligomannosidic glycans; furthermore, phosphoethanolamine or outer chains result in earlier elution, but galactofuranose and phosphorylcholine in higher retention.*
The use of hydrofluoric acid, a treatment known to release galactofuranose and phosphodiesterases (20, 22), as well as employing four types of α-mannosidase enabled us to demonstrate more than the overall composition of these glycans. In addition to short och1-dependent outer chains on the A-branch (including traces of glycans of up to Hexα2HexNAc2), we show the presence of galactofuranose as well as of zwitterionic modifications (phosphoethanolamine and phosphor-ylycholine). We assume that these three modifications of mannose residues are components not only of ‘releasable’ cellular proteins, but also of secreted glycoproteins and of the cell wall of these species. Indeed, galactofuranose as well as phosphodiester-linked ethanolamine and choline were previously found on a glycopeptide isolated from the peptidophosphogalactomannan of P. charlesii (29, 37).

Although the presence of galactofuranose on N-glycans from Eurotiomycetes has been previously shown on A. fumigatus secreted glycoproteins (38, 39), A. niger α-galactosidase (32) and Acremonium ascorbate oxidase (40), the locations (where defined) on the glycans are different as compared with the bisecting position on the core β1,4-mannose shown here; thus we consider bisecting galactofuranose to be a novel modification. Indeed, the β-galactofuranose on N-glycans from filamentous fungi is otherwise claimed to be on less “buried” locations such as a “replacement” for the B-mannose removed by endoplasmic reticulum mannosidase I (41) or on the core α1,6-mannose (40). Bisecting modifications of N-glycans from “lower” organisms are relatively rare, but bisecting β-GlcNAc is found in the slime mold Dictyostelium discoideum (15), bisecting α-GlcNAc in the basidiomycete Coprinopsis cinerea (42) and bisecting β1,4-galactose in Caenorhabditis elegans (43). The structural proof for galactofuranose on N-glycans from Penicillium, also as demonstrated by the GC-MS data, correlates with compositional analyses even 80 years ago and the occurrence of UDP-Gal, as a nucleotide sugar donor (44, 45).

An initially unexpected finding was the detection of phosphoethanolamine on N-glycans of Penicillium as well as low levels of phosphorylcholine in P. nordicum; such modifications are known on N-glycans of trichomonads, cestodes and nematodes (11, 46, 47) as well as on glycolipids of insects and nematodes (12, 48) and on lipopolysaccharides of some bacteria (10). The structural analysis suggests that sub-terminal positions on the A and C branches are apparently favored for the addition of phosphoethanolamine; this may explain why ManαGlcNAcβPE1 is underrepresented as compared with the occurrence of its neutral parent structure in P. dierckxii (compare Fig. 1A and 1D) or why there is no phosphoethanolamine-modified form of Man8C in P. nordicum (Fig. 3). Certainly, as compared with invertebrates, it is obvious that the zwitterionic modifications of Penicillium N-glycans are on mannose rather than on N-acetylgalactosamine residues. Interestingly, the Penicillium species with N-glycans carrying galactofuranose residues was the one with the least phosphoethanolamine as no more than a single such modification was found on N-glycans from P. verrucosum; thereby, phosphoethanolamine was not found on the α1,3-branch at all in this species, which may be an indication that the addition of galactofuranose may inhibit transfer of phosphoethanolamine or that the relevant transferase is not expressed. Indeed, differences in expression of phosphoethanolaminyltransferases may also explain the relatively high degree of modification of ManαGlcNAcβ with up to two phosphoethanolamine residues in P. dierckxii as compared with the other two species.

Biological Significance of Fungal Glycoepitopes—Galactofuranose, phosphoethanolamine and outer chain modifications are not present on mammalian N-glycans; thus, they may present potential antigenic epitopes or be ligands recognized by the innate immune system. As mentioned above, at least one Penicillium allergen displays carbohydrate-dependent IgE reactivity (4). On the other hand, an Aspergillus-reactive anti-galactofuranose antibody (used as an in vitro diagnostic) also recognizes Penicillium species (49) and it is of interest that galactofuranose-coated gold particles can interact with dendritic cells and thereby elicit an inflammatory response in a lectin-dependent manner (50) and that human interlectin-1 can bind β-galactofuranose (51). Furthermore, serum amyloid P protein (SAP), a mammalian pentraxin, known to recognize phosphoethanolamine, associates with components of the complement system (52); indeed, initial tests of Western blotting with SAP (followed by anti-SAP) showed binding to protein extracts of these Penicillium species. As Penicillium species are widespread in the environment and are also used in food production, the presence of galactofuranose and phosphoethanolamine on their N-glycans may well have relevance to the human immune system, particularly as infections with Penicillium species are an emerging problem in immunocompromised patients (53).

Glycogenomic Potential of the Eurotiomycetes—The basic progression of N-glycan biosynthesis in Penicillium species is expected to be similar to that of other fungi and yeasts, starting with the building up and transfer of GlcαManα-GlcNAcβ. However, there is relatively little specifically known about glycosylation pathways in Penicillium species, particularly as the Eurotiomycetes glycomutants known were all isolated from Aspergillus. It can, though, be assumed that during post-transfer processing, first glucosidases I and II remove the three glucose residues; deglucosylation seems to be relatively complete as only low amounts of glucosylated N-glycans were detected in the present study.

In terms of trimming by mannosidases, the so-called endoplasmic reticulum mannosidase I (the prototypic example being Mns1p from Saccharomyces cerevisiae (54)) removes the B-mannose; subsequently, class I mannosidases in the Golgi apparatus are known from many species (but not S. cerevisiae) which remove further mannose residues. Again the most information on filamentous fungi comes from the Aspergillus
genus, but a broad spectrum class I mannosidase capable of cleaving all α1,2-mannose residues from N-glycans is known from *Penicillium citrinum* (55). The action of this latter type of enzyme would account for the variable occurrence of Man$_{7-9}$GlcNAc$_2$ in all three *Penicillium* species examined here; however, in two of the three *Penicillium* glycomes (*P. nordicum* and *P. verrucosum*), the B isomer of Man$_8$GlcNAc$_2$ was underrepresented and indeed the frequent retention of the B α1,2-mannose in Man$_{6-8}$GlcNAc$_2$ (including the significant presence of Man8A in Man$_{6-8}$GlcNAc$_2$) suggests a relative lack of processing in vivo by endoplasmic reticulum α1,2-mannosidase I. On the other hand, the glycans from *P. dierckxii* tend to be smaller indicative of a higher degree of processing by class I mannosidases and so resulting in more Man$_{7-9}$GlcNAc$_2$ (including a higher amount of Man8B). A summary of the oligomannosidic isomers and their possible biosynthetic origin is shown in supplemental Fig. S9A.

As corroborated by the fragmentation patterns of mannosidase digestion products, all three species show the presence of short outer chains on the A branch of Man$_{7-9}$GlcNAc$_2$ base structures, whose synthesis is initiated by the Och1 α1,6-mannosyltransferase present in a wide variety of fungal and yeast species; glycans with longer Och1 arms were probably present, but could not definitively be analyzed because of low amounts. In terms of the Eurotiomycetes, *A. fumigatus* and *N. crassa och1* mutants were shown to have either altered secreted N-glycumes or altered cell walls (39, 56). Other potential Golgi mannosyltransferase homologues relevant to N-glycan modifications have also been noted in *Aspergillus* genomes (57, 58), whereas other mannan-synthesizing α-mannosyltransferases corresponding to Mnn1, Mnn2, Mnn5, and Mnn6 may be absent; the lack of such enzymes could account for the shorter chain lengths of N-glycans in Eurotiomycetes as compared with *S. cerevisiae*.

Mannosidase processing does not appear to be a prerequisite for the addition of galactofuranose or phosphoethanolamine, as Man9-based structures are also modified with these residues. There is a bias in addition of phosphoethanolamine addition toward the C branch, followed by a secondary preference for the A branch whereas modification of the B arm is rather seldom, which may correlate with the accessibility of the 6-hydroxyl functions of the subterminal mannose residues of the branches (for a simulated 3D-model of Man$_{1-3}$GlcNAc$_2$, refer to supplemental Fig. S9B). This is similar to the branch preference for addition of methylphosphate moieties in *Dictyostelium* (15). As mentioned above, outer chain mannosylation on the A branch is also found on the Man$_{8}$GlcNAc$_2$ base structure as well as on Man8A and Man8B isomers; it appears that the action of Och1p is not blocked if the endoplasmic reticulum Mns1p mannosidase has not acted on the B branch. This is in keeping with data on the *S. cerevisiae* mns1 mutant which still features outer chain mannosylation (59).

From our data, there is no evidence for intersecting α1,4-N-acetylglucosamine, peripheral α1,2-N-acetylglucosamine, α1,6-fucose, α1,2-galactose, α1,3-galactose, β1,2-mannose, pyruvylation of β3-galactose, or β1,2-xylose; these modifications have been found in other fungi and yeast (7, 8, 42, 60–64), but obvious orthologues of the relevant glycosyltransferase genes are seemingly absent from the Eurotiomycetes, although more distant members of, e.g. glycosyltransferase families GT8, GT31 and GT34 are present. Also phosphorylation (other than in the context of phosphoethanolamine) was apparently absent, which may correlate with the absence of obvious Mnn4/Pno1/Mpo1 phosphomannosyltransferase orthologues.

The best characterized genomes of Eurotiomycetes are those of some *Aspergillus* species, but only the glycogenome of one *Penicillium* species (P. chrysogenum, i.e. a species not included in our glycomic analysis) has been specifically catalogued in the CAZY database (65) with members of some thirty GT families being currently listed; further, members of four GH families relevant to N-glycan biosynthesis or processing are predicted. However, none of these proteins have been cloned as cDNAs so as to verify their actual sequence, never mind characterized in terms of enzyme activity or the impact of a mutation, but we can assume that many are involved in pathways of glycan and polysaccharide synthesis other than N-glycan modifications. Certainly, there is a wealth of potential glycosynthetic enzymes in filamentous fungi remaining to be discovered and whose characterization would aid the definition of enzyme specificities in many other species.

**CONCLUSION**

In this study we show a possibly unexpected complexity of the N-glycans of releasable cellular glycoproteins from three *Penicillium* species. Not only are there various isomers of oligomannosidic glycans, also with one or two residues indicating initiation of the ‘outer chain’, but we demonstrate up to three generally sub-terminal positions for the modification of mannose by phosphoethanolamine. Furthermore, not all hexose residues in these glycans are mannose, but galactofuranose is also present; interspecies variations also were found. This means that caution in terms of interpretation is required if only complete glycan profiling without fractionation or digestion is performed on glycans from fungi and yeast. It is to be expected that further variations will be uncovered as more species are subject to in-depth glycomic analyses. Thus, we conclude that fungal glycosylation will yield more surprises which may have repercussions for how we use these organisms as ‘factories’ for various biotechnological products.

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