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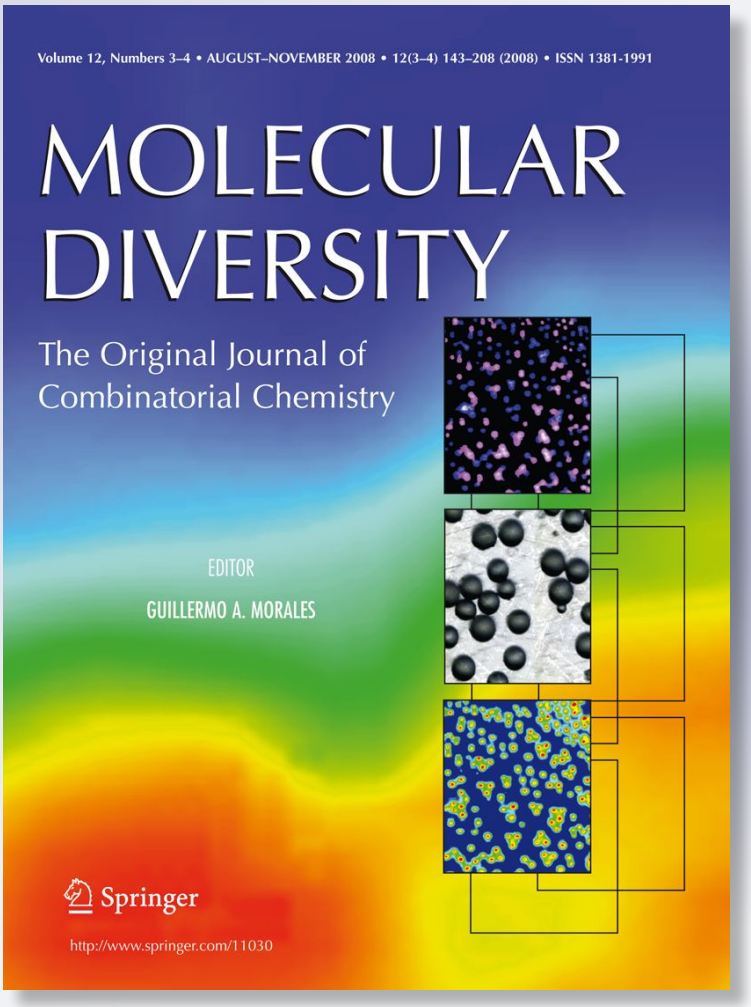

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FULL-LENGTH PAPER

Multicomponent synthesis of acylated short peptoids with antifungal activity against plant pathogens

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Abstract In this article, we describe the synthesis of a small library of short peptoids composed of four glycine residues and acylated with a fatty acid that showed a remarkable in vitro activity against two fungal plant pathogens. Their straightforward synthesis implied two consecutive Ugi reactions and can be efficiently extended to the construction of highly diverse libraries.

Keywords Acylated peptoids · Multicomponent reaction · Plant pathogen · Antifungal

Plants are constantly exposed to a variety of pathogenic microorganisms present in their environments. Diseases caused by pathogens, including bacteria, fungi, and viruses, significantly contribute to the overall loss in crop yield worldwide and constitute an emerging threat to the global food security [1]. Many of the currently available antimicrobial agents for agriculture are highly toxic and non-biodegradable and cause extended environmental pollution [2].

Antimicrobial peptides (AMPs) (12–50 amino acids long), known also as innate immunity host defense peptides or innate defense regulators, are key components of the innate immune system in all phyla, providing a fast-acting defense against invading pathogens [3,4]. A subfamily of AMPs with strong antimicrobial activity includes lipopeptides, which are produced non-ribosomally in bacteria and fungi. Lipopeptides consist of a short linear or cyclic peptide sequence to which a fatty acid moiety is covalently attached at the N terminus [5].

Recently, a new family of synthetic ultrashort lipopeptides (Fig. 1) composed of only four amino acid residues conjugated to long-chain aliphatic acids has been reported. These compounds have a broad spectrum of in vivo and in vitro antimicrobial activity against human-pathogenic yeasts, fungi, and bacteria [6], also affecting phytopathogenic fungi and bacteria [7].

Peptoids are a class of oligomeric N-substituted glycines that mimic the primary natural structure of peptides [8,9]. They are attractive non-natural molecules for drug discovery approaches because of their many biological activities and proteolytic stability. Many peptoids have been shown to be capable of acting as protein ligands with high affinity [10].

The most common method for the preparation of peptoids is the solid-phase submonomer procedure [11]. This method involves an iterative acylation reaction, with an R-haloacetyl moiety that is common to all backbone elongation processes and an iterative amination reaction using the commercially available primary amines, and has been recently employed to synthesize peptoid mimetics of antimicrobial lipopeptides [12]. An alternative approach involves the use of the Ugi four-component reaction (U-4CR) [13], which is a versatile tool for the construction of peptoid and mixed peptoid–peptide backbones. Repetitive or consecutive Ugi reactions have been used in the synthesis of peptide nucleic acid oligomers and in one-pot macrocyclizations [14,15]. However, the assembly of pure peptoid backbones in a consecutive fashion has not been extensively explored [16,17].

In this article, we describe the synthesis of a small library of N-substituted tetraglycines acylated with a long-chain alkyl residue. The structures resemble those of the antifungal

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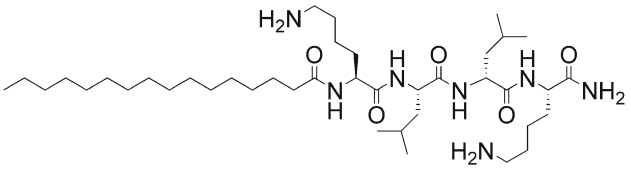
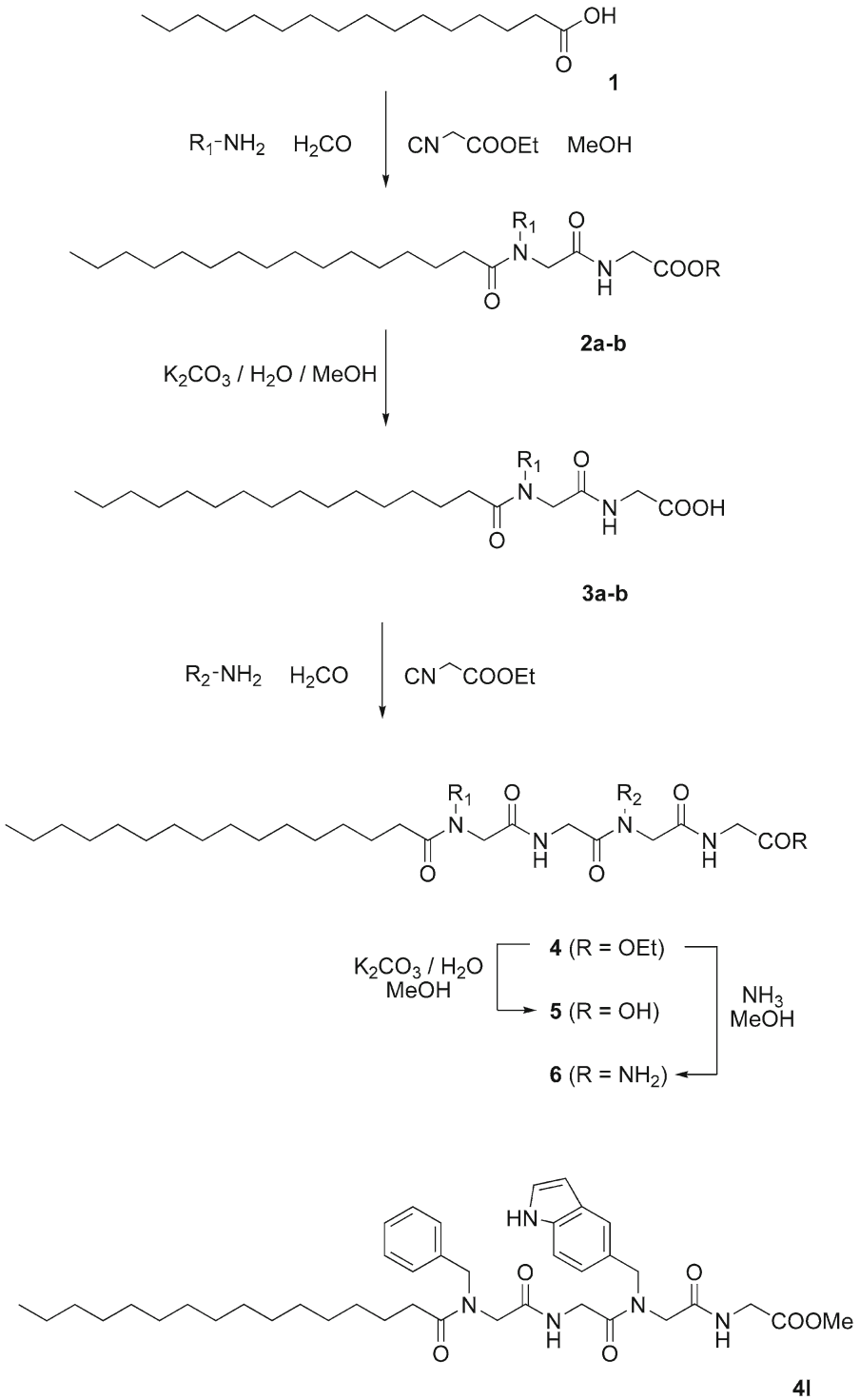


Fig. 1 A synthetic short lipopeptoid with antifungal activity (Ref. [6])

lipopeptides described by Makovitzki et al. [6], as they have a tetrapeptide backbone acylated with a palmitoyl residue, but are decorated with a more diverse set of side chains. A preliminary study on the antifungal properties of the new compounds against plant pathogens was carried out.

Scheme 1 Synthesis of new acylated peptoids



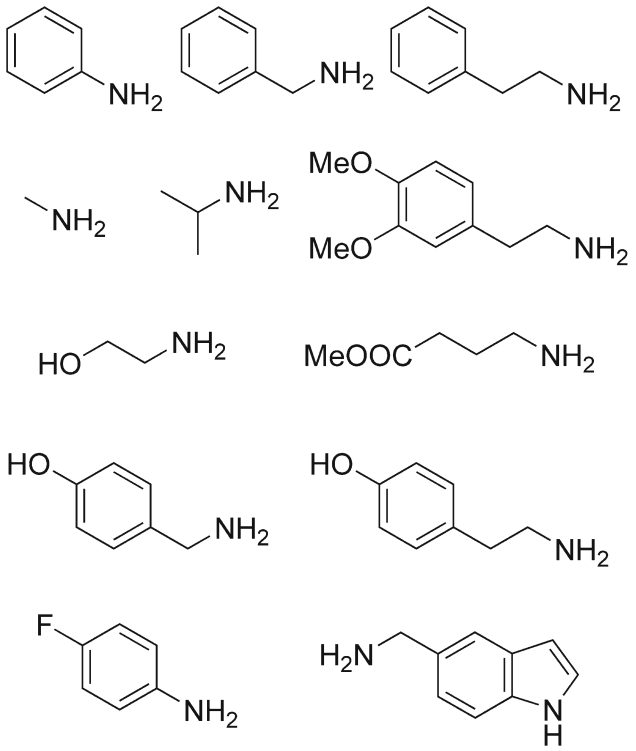


Fig. 2 Amines selected for the construction of the library

The strategy used to construct the tetraglycine backbone involved two consecutive Ugi reactions, as depicted in Scheme 1. The first Ugi reaction was performed using hexadecanoic acid (**1**), an amine (benzylamine or isopropylamine), formaldehyde, and ethyl isocyanoacetate. Thus, a diglycine, N-acylated with the desired fatty acid moiety (**2**), was obtained in one step. After completion of the reaction, intermediate esters of general structure **2** were hydrolyzed in situ without isolation to the resulting diglycines **3a**, **b** which were used in a subsequent Ugi reaction to give the acylated tetraglycines (12 compounds as ethyl esters of general

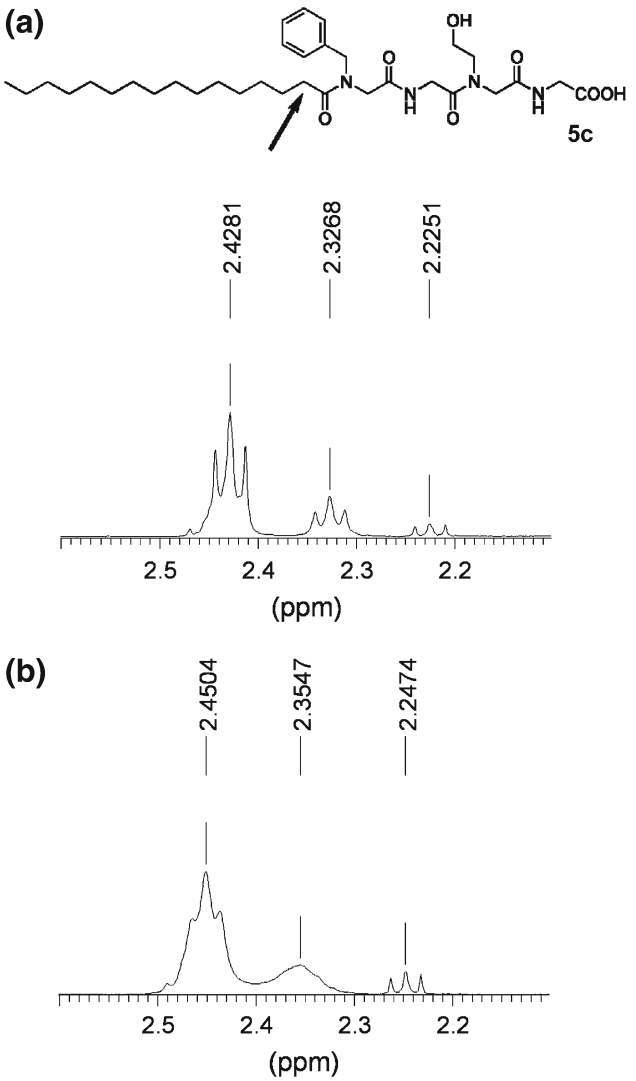
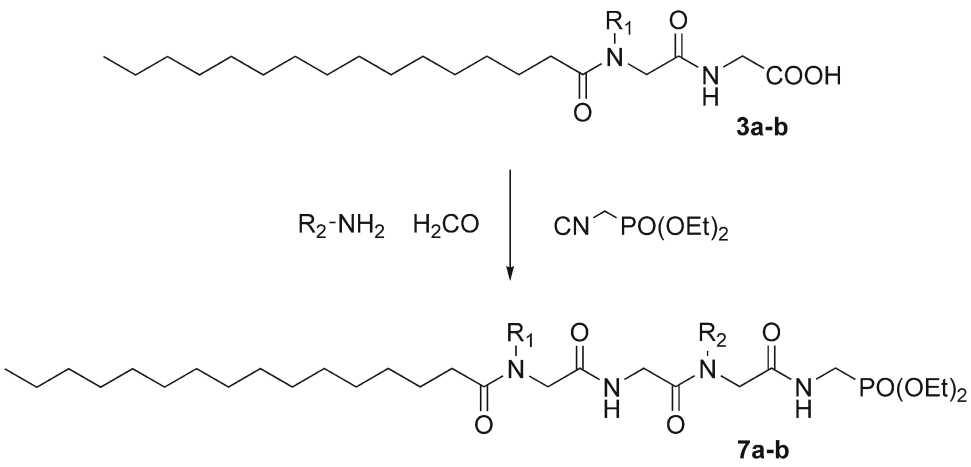


Fig. 3 Methylene signals (^{1}H-NRM, 500 MHz) for compound **5c** showing a mixture of conformers. Spectrum **a** recorded at 25 °C. Spectrum **b** recorded at 50 °C

Scheme 2 Synthesis of phosphonate analogs **7a** and **b**



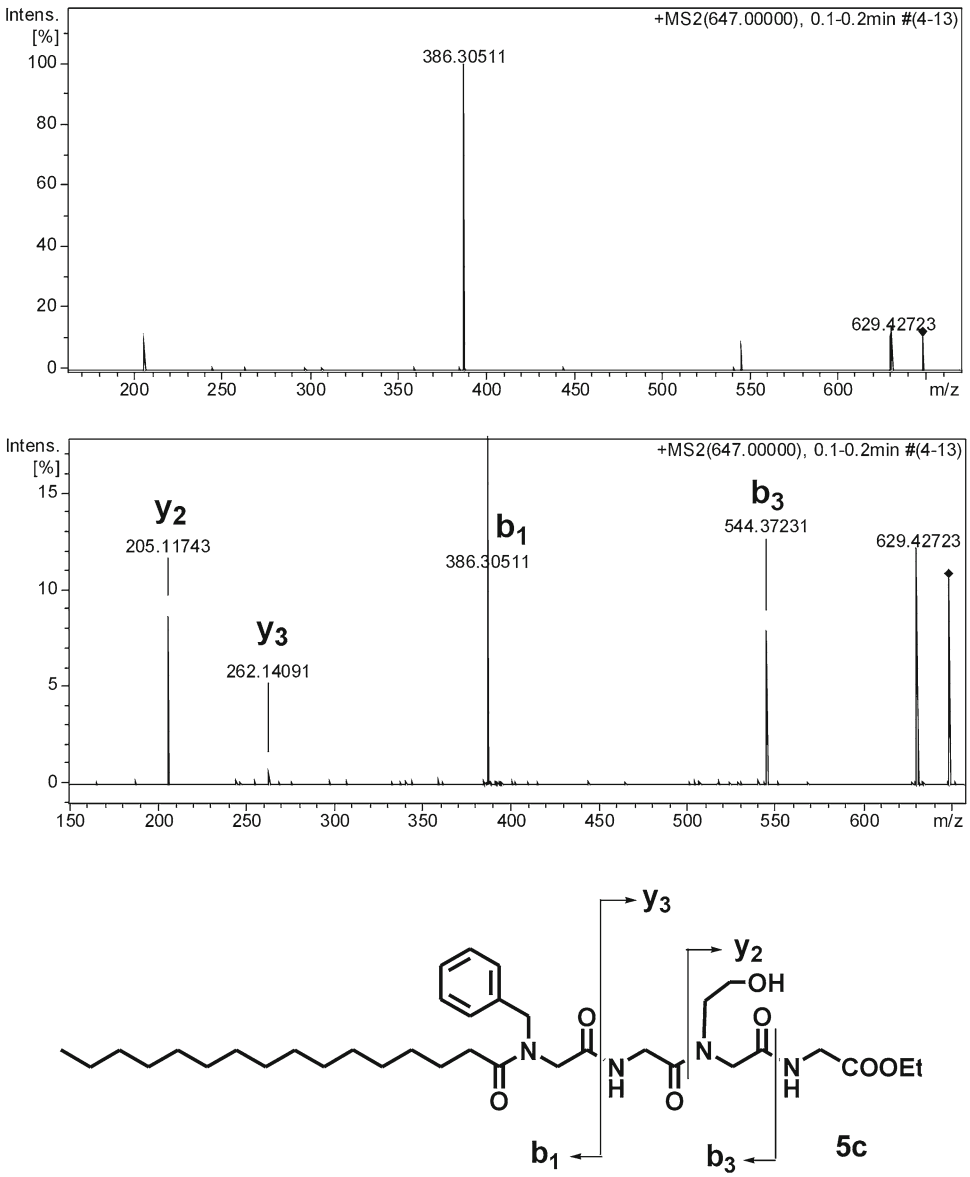


Fig. 4 ESI MS/MS spectrum of *m/z* 647 ($[M+H]^+$ cation of compound **5c** (collision Energy 10 eV) and peptoid backbone sequence ions

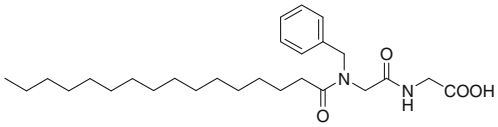
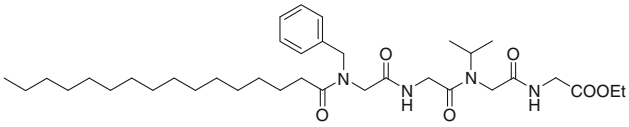
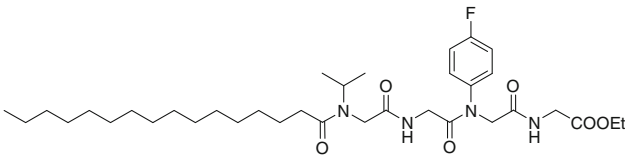
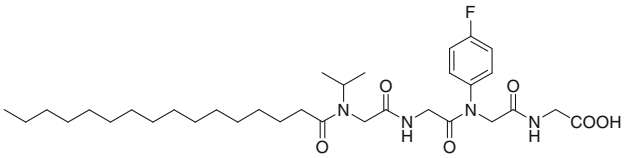
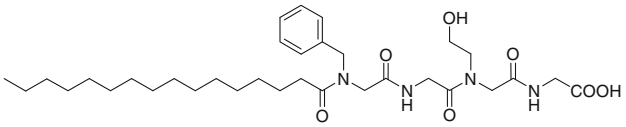
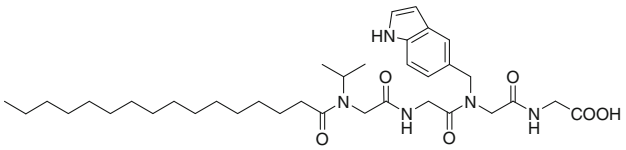
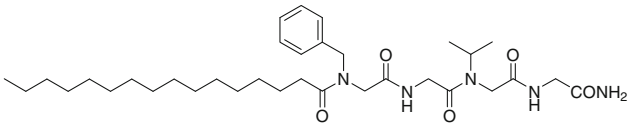
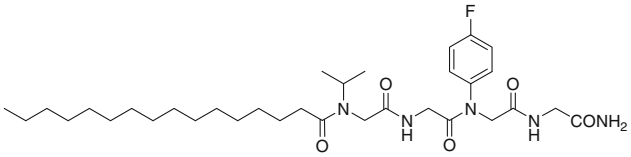
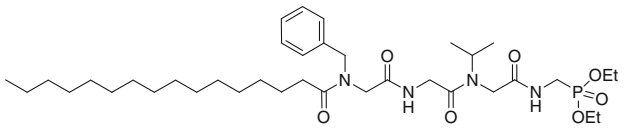
structure **4**), in high yields (> 80% for the two steps). Interestingly, one product was obtained as methyl ester (**4l**), which was probably formed by transesterification during the reaction (Scheme 1).

Both U-4CR took place smoothly and worked well with a set of structurally diverse amines (Fig. 2). Some of the amines were chosen to mimic the side chains of natural amino acids (e.g., methylamine, benzylamine, or *p*-hydroxybenzylamine, corresponding to alanine, phenylalanine, and tyrosine, respectively), while others, having additional functional groups, were used to increase the diversity.

Some of the esters obtained were alternatively hydrolyzed to yield the corresponding acids (**5**, 11 compounds) or treated with ammonia to give the amides **6** (10 compounds, Scheme 1). On the other hand, the acyldiglycyl acids **3** were used as substrates in an alternative U-4CR reaction which used isocyanomethyl diethylphosphonate as the isocyanide component, yielding the corresponding phosphonate analogs **7a**, **b** (Scheme 2). In total, a small library of 36 members was synthesized, and the compounds were purified by semi-preparative reverse-phase HPLC. The detailed experimental procedures are described in the Supplementary Material.

It is known that the nuclear magnetic resonance spectra of peptoids are very complex due to the occurrence of different conformations in solution. As an example, Fig. 3 shows the resonance assignment for the methylene adjacent to the carbonyl in compound **5c**, where at least three conformers are observed. The same spectrum was recorded

Table 1 Antifungal activities of selected compounds

		F. virguliforme [a]	*F.solani* [a]
3a		32	> 100
4a		23	> 100
4b		7	38
5b		8	> 100
5c		8	> 100
5d		15	23
6a		16	24
6b		16	> 100
7a		7	> 100

[a] Minimum inhibitory concentrations in μM. The MIC of the control (Benomyl, a commercial antifungal) is 2 μM for both fungi

at higher temperatures; the changes observed in the intensities and chemical shifts for these resonances are linked to a change in the conformer population, as previously reported [18]. The complexity of the spectra precluded their extensive use for the analysis of all new compounds. Thus, only the structures of the selected compounds were completely assigned by mono and bidimensional experiments.

In order to obtain a complete and unambiguous characterization of the library, we decided to perform a direct analysis of the purified peptoids via high resolution electrospray ionization mass spectrometry (HRMS ESI). In all cases, main peaks were observed for $[M+H]^+$, $[M+Na]^+$, and sometimes $[M+NH_4]^+$. Figure 4 shows a representative spectrum of peptoid **5c**. The molecular formula was shown to be $C_{35}H_{58}N_4O_7$ on the basis of its HRMS ESI

(m/z 647.4388 $[M+H]^+$, 664.4641 $[M+NH_4]^+$, and 669.4193 $[M+Na]^+$. The sequence of the amino acids in the peptoids was revealed by tandem mass spectrometry (MS/MS). For example, in the case of **5c**, the MS/MS of the $[M+H]^+$ ion, used as a precursor, yielded product ions m/z 544.3723, 386.3051, 262.1409, and 205.1174. These product ions can be assigned as ions b3, b1, y3, and y2 respectively, according to the nomenclature previously proposed by Heerma et al. [19], based on the Roepstorff nomenclature [20]. This procedure was applied to all the new compounds to determine their structure unambiguously (Supplementary Material).

Bioautography offers a rapid and convenient approach to identify novel antifungal compounds and requires only microgram quantities [21]. The new compounds were tested in vitro using a bioautography method for their inhibitory properties towards *Fusarium virguliforme* and *F. lateritium*. Most of the new compounds showed a measurable antifungal activity against either of the two fungi tested (See the Supplementary Material).

The minimum inhibitory concentrations (MIC's) of selected compounds against *F. virguliforme* and *F. solani* were assessed using a broth microdilution method [22], and are depicted in Table 1. These fungi are the casual agents of the sudden death syndrome, a disease of soybean that is spreading in important production areas.

Intriguingly, even the acylated diglycines (for example, **3a**) showed a remarkable activity, despite their simple structure.

Although this is a preliminary screening, the activity might be species-specific. In general *F. virguliforme* seemed more susceptible towards this family of compounds. On the other hand, the antifungal potency of the active compounds lies in the same range (6–50 μM) than those of the short synthetic lipopeptides described by Makovitzki et al. [23].

Natural lipopeptides have a membranolytic mode of action in fungi and bacteria [24], and the short synthetic analogues seem to act via the same mechanism. Interestingly, only cationic short lipopeptides exert inhibitory properties [23]. In contrast, the acylated peptoids described in this study are in most cases neutral or anionic, suggesting that an alternative mode of action might be operating.

In conclusion, we report on a new family of acylated short peptoids which show promising activities as antifungals against plant pathogens. Their straightforward synthesis and analysis are amenable to be extended to the preparation of larger and more diverse libraries.

Further study is under way to establish a structure–activity relationship for these compounds and their possible mechanism of action on fungal growth.

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