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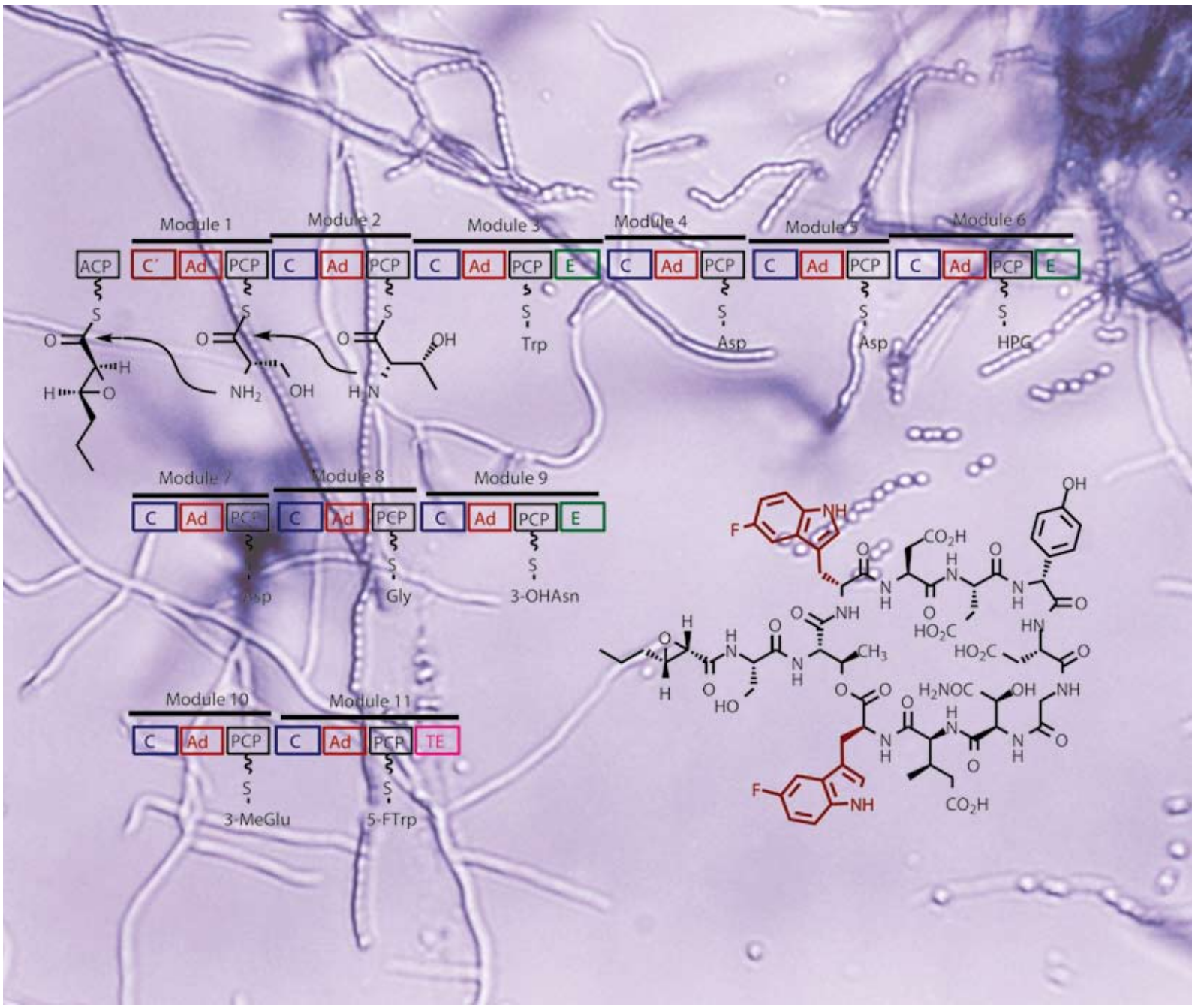

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Auxotrophic-precursor directed biosynthesis of nonribosomal lipopeptides with modified tryptophan residues†

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Feeding 5-hydroxy and 5-fluorotryptophan to a *Streptomyces coelicolor* Trp-auxotrophic strain WH101 results in the production of a number of new calcium-dependent antibiotics (CDAs) possessing modified Trp residues. It is anticipated that this method could be used to modulate the biological properties of Trp-containing nonribosomal peptide natural products, or to generate analogues with useful fluorescent properties for studying biological mechanisms of action.

For many years auxotrophic strains of bacteria and fungi, which are unable to produce a specific proteinogenic amino acid, have been exploited to produce proteins containing amino acid analogues.[1] For example tryptophan analogs including 7-azatryptophan (7AW) **1** and 5-hydroxytryptophan (5HW) **2** (Fig. 1) have been incorporated into proteins in Trp-auxotrophic bacteria, grown on minimal media with depleted levels of L-Trp.[2] Such Trp analogues (**1** and **2**) have distinct fluorescence properties, which can be used to probe the structure, function and dynamics of selected proteins.[2] Indeed shorter peptides (domains) have been similarly produced, possessing modified Trp residues, and then subsequently assembled into larger multidomain proteins using expressed protein ligation.[2c] The biological properties of small ribosomally encoded peptides, including the disulfide bridged cyclic peptide compstatin, can also be improved by incorporation of Trp analogues using an *E. coli* Trp-auxotroph.[3] Finally, precursor directed biosynthesis, involving feeding amino acid analogues to wild-type bacterial and fungal strains, has also resulted in the production of secondary metabolite analogues incorporating modified amino acid residues.[4] For example fluorinated analogs of the nonribosomal peptide actinomycin D were produced by feeding 5-fluorotryptophan (5FW) **3** to *Streptomyces parvullus*.[4b]

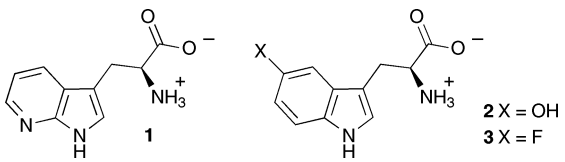

Fig. 1 Structures of 7-azatryptophan **1**, 5-hydroxytryptophan **2** and 5-fluorotryptophan **3** analogues used in this study.

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Despite this, there are very few examples where amino acid auxotrophs have been exploited in the precursor directed biosynthesis of natural products.[5]

Previously, we used the Trp-auxotrophic WH101 strain of *Streptomyces coelicolor*[6] to determine the stereochemical course of Trp dehydrogenation during the biosynthesis of *Z*-2′,3′-dehydrotryptophan (*Z*-ΔTrp) found at the C-terminal position 11 of the calcium-dependent antibiotics (CDA) (Fig. 2).[7] CDA is a nonribosomal lipopeptide, which in addition to *Z*-ΔTrp, contains a number of other nonproteinogenic and D-amino acids, including D-Trp at position 3, as well as an N-terminal *trans*-2,3-epoxyhexanoyl fatty acid side chain.[8] CDA shares a similar structure, and probably a related calcium-dependent antimicrobial mechanism of action, as the commercial lipopeptide antibiotic daptomycin.[9] As a result of this, there has been considerable interest in developing new methods for the engineered, and precursor directed, biosynthesis of new lipopeptide variants.[10] Following on from this, it was envisaged that the *S. coelicolor* WH101 strain, which can incorporate high levels (*ca.* 60%) of deuterated Trp into CDA when grown on minimal medium,[7] might be used in the directed biosynthesis of CDAs possessing modified Trp-derived residues.

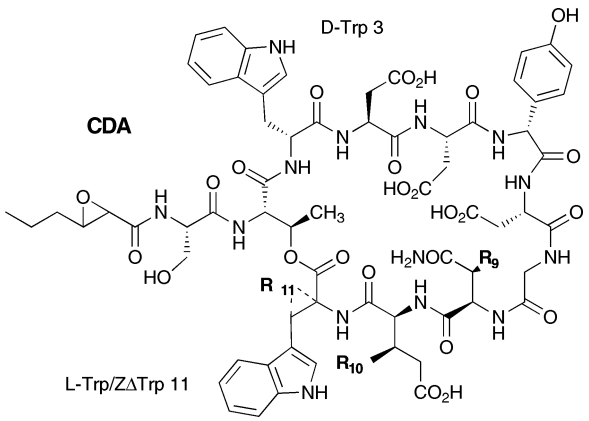

Fig. 2 Calcium-dependent antibiotics (CDAs) produced by the wild type *S. coelicolor*: CDA1, $R_9 = OPO_3H_2$ and $R_{10} = H$; CDA2, $R_9 = OPO_3H_2$ and $R_{10} = CH_3$; CDA3, $R_9 = OH$, $R_{10} = H$; CDA4, $R_9 = OH$, $R_{10} = CH_3$. The a-series contain *Z*-ΔTrp ($R_{11} = \pi$-bond) and the b-series contain L-Trp ($R_{11} = H,H$) at position 11.

Accordingly, the WH101 strain was grown in the minimal medium SV2a, estimated to contain low levels of L-Trp (*ca.* 6.0 μg mL^{-1}),[7] which was supplemented with D/L-7-azatryptophan (7AW) **1** (75.0 μg mL^{-1}). Following fermentation, the culture supernatants were analysed by ESI-LC-MS.[7] Detailed analysis of the LC-MS failed to show the presence of any CDAs with

molecular weights consistent with 7-azatryptophan containing variants. Instead, wild-type CDA4b was shown to be the major product by comparison with an authentic sample. In the absence of 7AW **1**, the WH101 strain, under identical conditions, produces predominantly the a-series CDA4a with a minor amount of CDA3a, which both contain C-terminal *Z*-ΔTrp residues. Thus, whilst 7AW **1** is not incorporated into CDA, it apparently inhibits the production of a-series CDA in favour of CDA4b, which possesses a C-terminal L-Trp, rather than a *Z*-ΔTrp residue. One possible reason for this could be that 7AW inhibits the putative Trp dehydrogenase/oxidase enzyme that is predicted to catalyse the oxidation of the C-terminal Trp side chain.[7] Indeed, Trp analogues with modifications in the indole ring have been shown to inhibit the L-tryptophan oxidase (LTO) from *Chromabacterium violaceum*,[11] which shares some mechanistic similarity to the enzyme responsible for *Z*-ΔTrp in CDA.[7]

Following this, *S. coelicolor* WH101 was grown in SV2a, supplemented with 5-hydroxytryptophan (5HW) **2** (37.5 μg mL^{-1}), and the culture supernatants were analysed by LC-MS (Fig. 3). This revealed that CDA4a was the major product (R_t = 6.98). However, in addition, three new products were evident that are not produced by the WH101 strain in the absence of 5HW **2**. Furthermore, these new products do not correspond with the retention times or MS molecular ions that are observed for any of the known CDAs produced by *S. coelicolor*. Indeed, two of the products (R_t = 5.96 and 6.19 min) exhibit molecular ions that are exactly 16 Da higher than CDA4a. This is consistent with the single incorporation of 5HW **2** at either position 3 or 11 of CDA4a. The low quantities of these products and difficulties associated with their separation prevented isolation of sufficient quantities of peptides for the assignment of the regiochemistry of the 5HW incorporation. However, a fourth additional minor product was also evident with a shorter retention time (R_t = 5.19 min) and molecular ions that are 32 Da higher than CDA4a. The increased polarity and MS of this product is thus consistent with a new CDA analogue that has incorporated two 5HW molecules at both position 3 and 11 of CDA4a. Furthermore, the molecular ions of the three new products in this case are all consistent with 2′,3′-dehydrogenation of the Trp or 5HW residues, at position 11, having taken place. This suggests that the putative CDA Trp dehydrogenase/oxidase biosynthetic enzyme is not inhibited by 5HW and indeed can accept a 5HW residue as a substrate, presumably oxidising this to the 2′,3′-dehydro-5-hydroxytryptophan residue.

In addition to this, 5-fluorotryptophan (5FW) **3** (37.5 μg mL^{-1}) was fed to the WH101 auxotroph in a similar fashion. In this case, there is evidence of very low-level production of CDA4b and CDA3b, however, none of the a-series wild-type lipopeptides is observed above the threshold level of detection. Instead, the major products from these feedings are a group of four closely eluting compounds, which are clearly not evident in fermentations in the absence of 5FW (Fig. 4). In addition, these products have longer retention times and do not possess molecular ions in the MS that correspond with any known CDAs produced by WH101 or any other known *S. coelicolor* strain. The products with retention times of 7.41 and 7.52 min, exhibit molecular ions that are consistent with the single incorporation of 5FW into CDA3b and CDA4b, respectively. In addition, the two remaining products with longer retention times of 7.62 and 7.71 min, exhibit molecular ions in the

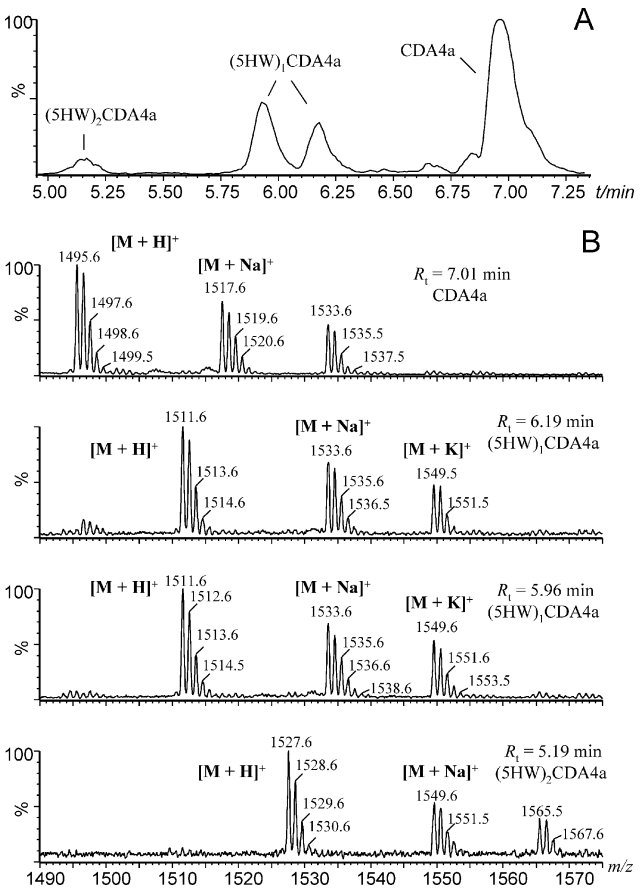

Fig. 3 (**A**) HPLC trace and (**B**) MS of the products resulting from feeding 5-hydroxytryptophan (5HW) **2** to *S. coelicolor* WH101. R_t = 7.01 min, CDA4a: *m/z* 1495.6 ([M + H]$^+$ $C_{67}H_{79}N_{14}O_{26}$ requires 1495.5); 1517.6 ([M + Na]$^+$ $C_{67}H_{78}N_{14}O_{26}$Na requires 1517.5); 1533.6 ([M + K]$^+$ $C_{67}H_{78}N_{14}O_{26}$K requires 1533.5). R_t = 6.19 and 5.96 min, single incorporation (5HW) CDA4a regioisomers: *m/z* 1511.6 ([M + H]$^+$ $C_{67}H_{79}N_{14}O_{27}$ requires 1511.5); 1533.6 ([M + Na]$^+$ $C_{67}H_{78}N_{14}O_{27}$Na requires 1533.5); 1549.6 ([M + K]$^+$ $C_{67}H_{78}N_{14}O_{27}$K requires 1549.5). R_t = 5.17 min, double incorporation product, $(5HW)_2$CDA4a: *m/z* 1527.6 ([M + H]$^+$ $C_{67}H_{79}N_{14}O_{28}$ requires 1527.5); 1549.6 ([M + Na]$^+$ $C_{67}H_{78}N_{14}O_{28}$Na requires 1549.5); 1565.6 ([M + K]$^+$ $C_{67}H_{78}N_{14}O_{28}$K requires 1549.5).

MS that are consistent with the double incorporation of 5FW into both positions 3 and 11 of CDA3b and CDA4b, respectively. Given that the incorporation of 5FW **3** into CDA is apparently higher than 5HW **2**, this would appear to suggest that the 5FW is a better substrate for the NRPS enzymes that activate and condense the Trp precursors. On the other hand, the absence of any wild-type a-series CDA or any probable a-series CDA variants possessing 5FW residues suggests that 5FW residues are not substrates for the Trp-dehydrogenase/oxidase and probably 5FW also inhibits the dehydrogenation of the natural Trp residues in the wild-type CDAs.

Conclusion

In summary, using the Trp-auxotrophic *S. coelicolor* WH101 strain, it is possible to effect the precursor directed biosynthesis

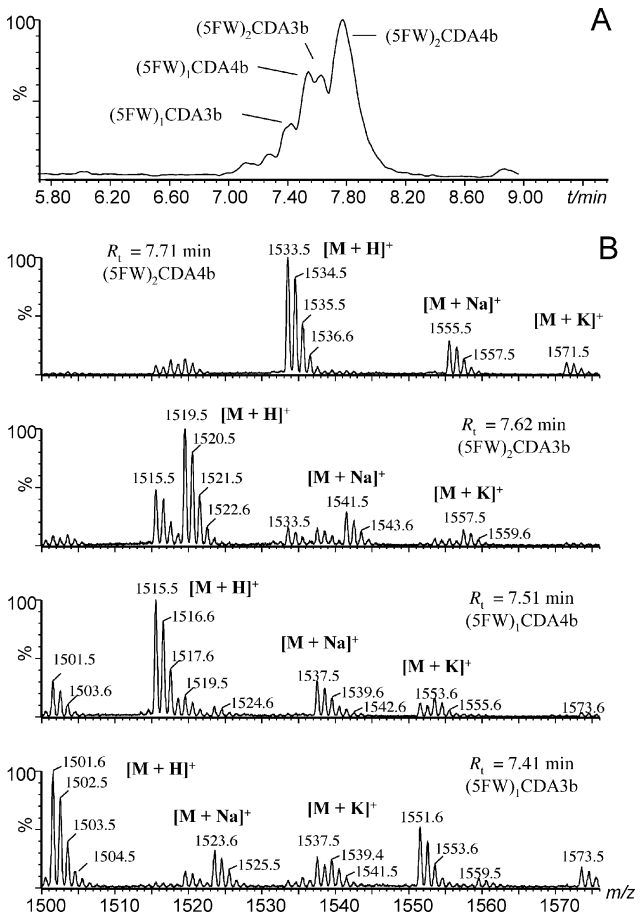

Fig. 4 (**A**) HPLC trace and (**B**) MS of the products resulting from feeding 5-fluorotryptophan (5FW) **3** to *S. coelicolor* WH101. R_t = 7.71 min, double incorporation product $(5FW)_2CDA4b$: *m/z* 1533.5 ($[M + H]^+$ $C_{67}H_{79}F_2N_{14}O_{26}$ requires 1533.5), 1555.5 ($[M + Na]^+$ $C_{67}H_{78}F_2N_{14}Na_1O_{26}$ requires 1555.5), 1571.5 ($[M + K]^+$ $C_{67}H_{78}F_2K_1N_{14}O_{26}$ requires 1571.5); R_t = 7.62 min, double incorporation product $(5FW)_2CDA3b$: *m/z* 1519.5 ($[M + H]^+$ $C_{66}H_{77}F_2N_{14}O_{26}$ requires 1519.5), 1541.5 ($[M + Na]^+$ $C_{66}H_{76}F_2N_{14}Na_1O_{26}$ requires 1541.5), 1557.5 ($[M + K]^+$ $C_{66}H_{76}F_1K_1N_{14}O_{26}$ requires 1557.5); R_t = 7.51 min, single incorporation product $(5FW)_1CDA4b$: *m/z* 1515.5 ($[M + H]^+$ $C_{67}H_{80}F_1N_{14}O_{26}$ requires 1515.5), 1537.5 ($[M + Na]^+$ $C_{67}H_{79}F_1N_{14}Na_1O_{26}$ requires 1537.5), 1553.6 ($[M + K]^+$ $C_{67}H_{79}F_1K_1N_{14}O_{26}$ requires 1537.5); R_t = 7.41 min, single incorporation product $(5FW)_1CDA3b$: *m/z* 1501.6 ($[M + H]^+$ $C_{66}H_{78}F_1N_{14}O_{26}$ requires 1501.5); 1523.6 ($[M + Na]^+$ $C_{66}H_{77}F_1N_{14}Na_1O_{26}$ requires 1523.5), 1539.4 ($[M + K]^+$ $C_{66}H_{78}F_1K_1N_{14}O_{26}$, requires 1539.5).

of CDA analogues possessing modified Trp residues by feeding 5-hydroxy and 5-fluorotryptophan analogues (**2** and **3**). Interestingly, the 7-azatryptophan analogues cannot be incorporated into CDA in this way. The typical low yields for production of CDAs and difficulties associated with separating the products, including regioisomers, complicated by the fact that CDA possesses two Trp derived residues, prevented the isolation of sufficient quantities of products for biological testing. Nevertheless, it is possible that this approach might be used to modify Trp residues found in other natural products, particularly nonribosomal peptides, which could lead to new products with improved biological activities. For example, the commercial lipopeptide daptomycin, which was approved for use in 2003 and is now widely used in the clinic, possesses an L-Trp residue as well as a Trp derived L-kynurenine (Kyn) residue. Indeed, the *Streptomyces roseosporus* industrial daptomycin production strain gives *ca.* 100 fold higher titres of lipopeptides compared to *S. coelicolor.* It is thus likely that a *S. roseosporus* Trp-auxotroph could be used to produce significant quantities of daptomycins possessing modified Trp and Kyn residues. In addition to potentially improving the biological properties of the lipopeptide, those analogs possessing 5HW will exhibit fluorescence excitation at wavelengths longer than the absorbance of Trp and thus their fluorescence can be selectively excited.[2a] The distinct fluorescent properties of such lipopeptides could thus be used to further probe the structure and dynamics of lipopeptides. In addition, fluorescence could be used to study the interactions of lipopeptides with specific molecular targets within bacterial cell membranes. This could assist in efforts to further elucidate the antimicrobial mechanisms of action of these lipopeptides, which remains a subject of considerable debate and uncertainty.[9,12]

Acknowledgements

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