ChemPubSoc Europe

DOI: 10.1002/chem.201604279

CHEMISTRY A European Journal Full Paper

Medicinal Chemistry

Total Synthesis of Dansylated Park's Nucleotide for High-Throughput MraY Assays

Stephanie Wohnig,[a] Anatol P. Spork,[a, b] Stefan Koppermann,[a] Gottfried Mieskes,[b] Nicolas Gisch,[c] Reinhard Jahn,[b] and Christian Ducho*[a]

In memory of Professor Chris McGuigan (1958–2016)

Abstract: The membrane protein translocase I (MraY) is a key enzyme in bacterial peptidoglycan biosynthesis. It is therefore frequently discussed as a target for the development of novel antibiotics. The screening of compound libraries for the identification of MraY inhibitors is enabled by an established fluorescence-based MraY assay. However, this assay requires a dansylated derivative of the bacterial biosynthetic intermediate Park's nucleotide as the MraY substrate. Isolation of Park's nucleotide from bacteria and subsequent dansylation only furnishes limited amounts of this substrate, thus hampering the high-throughput screening for MraY inhibitors. Accordingly, the efficient provision of dansylated Park's nucleotide is a major bottleneck in the exploration of this promising drug target. In this work, we present the first total synthesis of dansylated Park's nucleotide, affording an unprecedented amount of the target compound for high-throughput MraY assays.

Introduction

Bacterial strains with resistances towards established antibiotics continue to emerge, thus representing a major threat to human health.[1] The development of novel antibiotics is therefore highly desirable. In order to circumvent existing resistance mechanisms, such novel antimicrobial agents should ideally display new modes of action.

Many clinically used antibiotics, such as the β-lactams or vancomycin,[2] block the formation of the bacterial cell wall structure by inhibition of peptidoglycan biosynthesis. However, almost all of these therapeutic agents inhibit one of the late extracellular steps of peptidoglycan biosynthesis, while the early intracellular steps remain largely unexplored. The bacterial membrane protein MraY is one of the key enzymes in the intracellular section of peptidoglycan formation and is frequently mentioned as a potential new drug target.[3] MraY catalyzes the reaction of the cytosolic precursor UDP-Mur*N*Ac-pentapeptide ("Park's nucleotide") **1** with the membrane anchor undecaprenyl phosphate, thus furnishing the membrane-bound biosynthetic intermediate lipid I (**2**; Scheme 1).[4]

According to a topology model, MraY was predicted to be an integral membrane protein comprised of ten transmembrane helices and five cytoplasmic loops forming the active site.[5a] This principle architecture was recently confirmed by the first X-ray crystal structure of an MraY homologue, in this case from the extremophile *Aquifex aeolicus*.[5b] Mechanistic studies on MraY including mutagenesis were also performed, but have not provided a universally accepted molecular mechanism of the enzyme's catalytic action yet.[6] Several MraY homologues from different bacterial species were heterologously overexpressed, including cell-free expression methodology.[7]

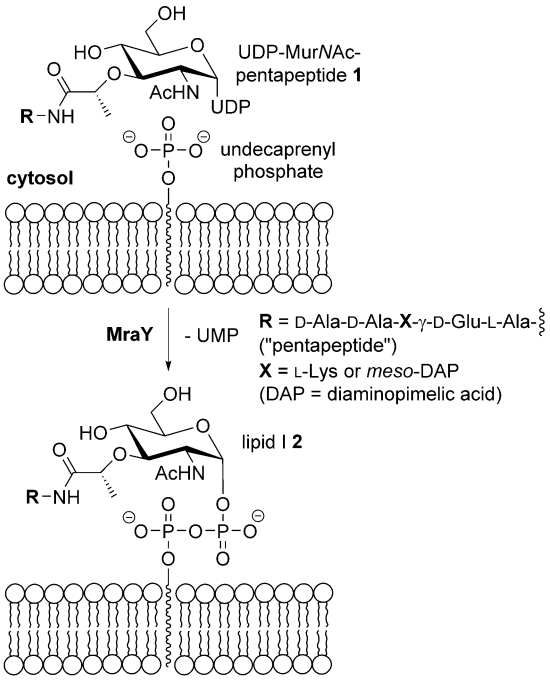
Naturally occurring uridine-derived nucleoside antibiotics (e.g., muraymycins, caprazamycins, liposidomycins, capuramycins and mureidomycins) and their analogues are known to be potent inhibitors of MraY.[8] So far, insights into the molecular details of MraY inhibition have mainly been based on structure–activity relationship (SAR) data (for selected examples for muraymycins see ref. [9]) and also on some biochemical studies.[10] Very recently, the first X-ray co-crystal structure of MraY from *Aquifex aeolicus* in complex with an inhibitor, that is, the natural product muraymycin D2, has been reported.[11] A comparison of this enzyme–inhibitor complex with the structure of the apo-protein[5b] revealed that MraY undergoes significant conformational changes upon ligand binding. This pronounced conformational plasticity suggests that computer-aided drug

[a] Dr. S. Wohnig, Dr. A. P. Spork, S. Koppermann, Prof. C. Ducho
Department of Pharmacy, Pharmaceutical and Medicinal Chemistry
Saarland University, Campus C2 3, 66123 Saarbrücken (Germany)
and
Department of Chemistry, Institute of Organic and Biomolecular Chemistry
Georg-August-University Göttingen, Tammannstr. 2
37077 Göttingen (Germany)
E-mail: christian.ducho@uni-saarland.de

[b] Dr. A. P. Spork, Dr. G. Mieskes, Prof. R. Jahn
Max-Planck-Institute for Biophysical Chemistry
Am Fassberg 11, 37077 Göttingen (Germany)

[c] Dr. N. Gisch
Division of Bioanalytical Chemistry, Research Center Borstel
Leibniz-Center for Medicine and Biosciences
Parkallee 1–40, 23845 Borstel (Germany)

Supporting information and ORCIDs from the authors for this article are available on the WWW under http://dx.doi.org/10.1002/chem.201604279.



Scheme 1. MraY-catalyzed reaction of Park's nucleotide **1** towards lipid I (**2**; undecaprenyl phosphate represented schematically; UDP = uridine diphosphate; UMP = uridine monophosphate).

design based on the structure of the MraY–inhibitor complex will be challenging. It is therefore expected that the identification of novel MraY inhibitors, particularly synthetic non-nucleoside congeners, will be based on the screening of compound libraries using high-throughput MraY assays in vitro.

Several assays are established for in vitro studies on MraY activity (and therefore also its inhibition). In principle, three different methods have been reported so far: 1) a fluorescence-based assay established by Bugg et al.;[12] 2) a radioactivity-based assay;[9a,12c] 3) a Förster resonance energy transfer (FRET)-based assay.[13] Comparing these options, Bugg's fluorescence-based assay (1) appears to be particularly useful as it has been shown to provide robust results and to be compatible with high-throughput screening (HTS) methodology.[12c] Bugg's assay (1) employs dansylated Park's nucleotide **3** (with the dansyl moiety attached to the lysine side chain) as a substrate analogue (Figure 1). The incubation of **3** with recombinantly expressed MraY and commercially available undecaprenyl phosphate in the presence of Mg^{II} furnishes dansylated lipid I (structure not shown), which displays an increased fluorescence relative to **3**. Thus, the assay mixture shows an increase of fluorescence over time, which can be slowed down or blocked by addition of an MraY inhibitor.

A potential application of the fluorescence-based assay for the HTS of larger compound libraries would require a robust supply of significant amounts of the dansylated substrate **3**. Previously, **3** was obtained by semi-synthetic dansylation of Park's nucleotide **1** isolated from bacterial cultures.[12c] The obvious disadvantage associated with this method consists in the intrinsically low output of **1**. We have therefore decided to

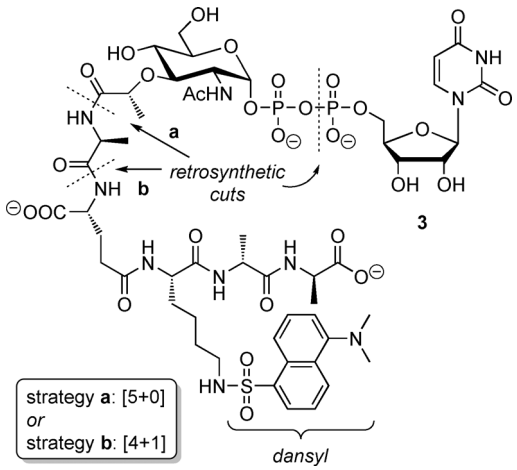


Figure 1. Dansylated Park's nucleotide **3**, the substrate for the fluorescence-based high-throughput MraY assay and target structure of this study, as well as potential retrosynthetic strategies.

pursue a total chemical synthesis of **3** with its performance to be benchmarked against the productivity of the semi-synthetic approach.

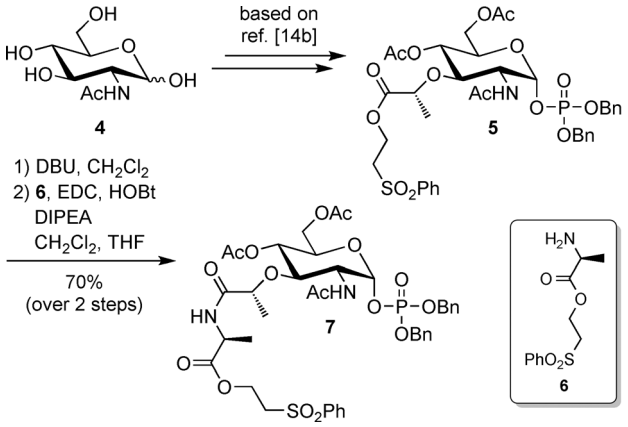
Results and Discussion

While the preparation of **3** by total synthesis is unprecedented, several syntheses of either Park's nucleotide **1** or related congeners were reported before.[14] Hitchcock et al. were the first to obtain **1** by total chemical synthesis.[14b] In contrast, both Wong et al.[14c] and Kurosu et al.[14d] have established chemoenzymatic methodology to afford **1** and other UDP-*N*-acetylmuramyl-peptides. Following work by Li and Kurosu,[14e] Wong et al. have further reported a chemical synthesis of *N*-glycolyl Park's nucleotide (the according peptidoglycan precursor in *Mycobacterium tuberculosis*)[14f] as part of their work on biocatalytic routes towards lipid I and lipid II derivatives.[14f,g]

In order to avoid any complications associated with the overexpression and purification of biosynthetic enzymes, we have decided to develop a chemical synthesis of **3**, excluding chemoenzymatic procedures. The chemical syntheses of Hitchcock[14b] and Kurosu/Wong[14e,f] have in common the fact that the UDP moiety is formed at a late stage by transformation of a glycosyl monophosphate precursor. Remarkably, their strategies differ in the construction of the pentapeptide moiety. Hitchcock has assembled a complete pentapeptide building block and coupled it to a protected *N*-acetyl muramic acid (Mur*N*Ac) derivative, while both Kurosu and Wong have connected a tetrapeptide unit to a Mur*N*Ac-L-Ala building block. With respect to the formal retrosynthetic cuts relative to the pentapeptide structure, we will label the former approach (strategy a, Figure 1) as "[5+0]" and the latter route (strategy b, Figure 1) as "[4+1]". For our total synthesis of **3** reported herein, we have comparatively studied both strategies for the assembly of the Mur*N*Ac-pentapeptide unit.

Our synthesis commenced with the preparation of the protected Mur*N*Ac derivatives. Starting from *N*-acetyl-glucosamine

(Glc*N*Ac) **4**, we have employed Hitchcock's route to synthesize protected Mur*N*Ac phosphate **5**, that is, the key building block for the [5+0]-strategy (Scheme 2). While we followed the principle route reported by Hitchcock et al.,[14b] considerable variations of several reaction conditions were required in order to achieve satisfactory yields (details are given in the Supporting Information). The protected Mur*N*Ac derivative **5** was then further converted into the corresponding carboxylic acid by cleavage of the phenylsulfonylethyl ester under non-nucleophilic conditions (DBU in dichloromethane). Subsequent esterification with L-alanine phenylsulfonylethyl ester **6** furnished protected Mur*N*Ac-L-Ala phosphate **7**, that is, the key intermediate for the [4+1]-strategy, in 70% yield over 2 steps from **5** (Scheme 2).



Scheme 2. Synthesis of the protected Mur*N*Ac phosphate building blocks **5** and **7**. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, HOBt = 1-hydroxybenzotriazole.

For the assembly of the peptide unit, D-alanine methyl ester hydrochloride **8** and *N*-Cbz-D-alanine **9** underwent peptide coupling (product **10**, 85% yield), followed by hydrogenolytic Cbz deprotection in quantitative yield to give the ester-protected D-Ala-D-Ala dipeptide **11** (Scheme 3). The hydrogenolysis reaction was carried out in the presence of TFA in order to isolate the resultant amine as its TFA salt, thus preventing unwanted diketopiperazine formation by intramolecular reaction of the amino group with the methyl ester.

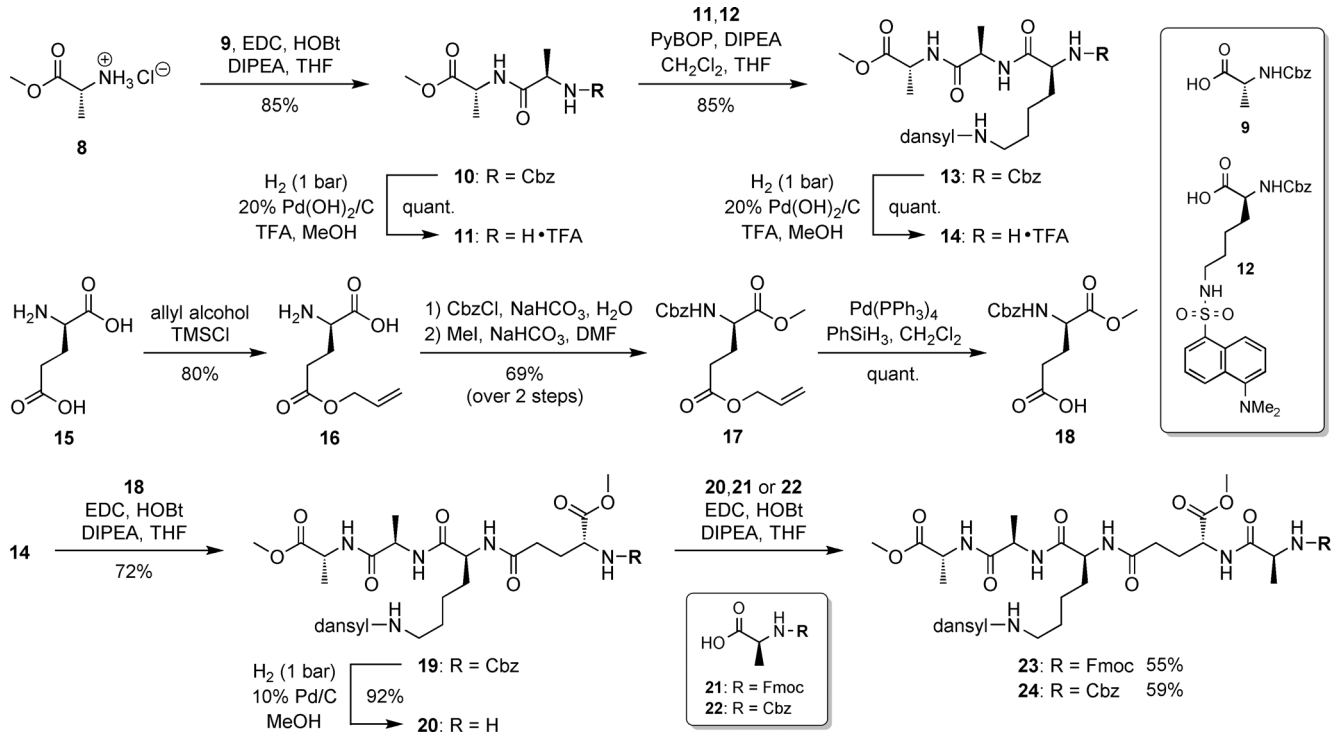
The next coupling step towards a tripeptide intermediate involved a lysine building block. In order to avoid an additional protection/deprotection sequence for the lysine N^{ε}-amino group, we desired to introduce the dansyl unit at an early stage. Therefore, we adapted the previously reported synthesis of N^{α}-Cbz-N^{ε}-dansyl-L-lysine **12**[15] with some slight modifications (see the Supporting Information) and coupled **12** with dipeptide **11** (Scheme 3). Several reagents were studied for this coupling step, with PyBOP giving the best results. Thus, protected tripeptide **13** was obtained in 85% yield and its Cbz-deprotected congener **14** (as a TFA salt) after hydrogenolysis in quantitative yield.

Subsequent formation of the tetrapeptide required a suitably protected derivative of D-glutamate **15**. Regioselective side chain esterification of **15** was achieved in the presence of trimethylsilyl chloride,[16] affording allyl ester **16** in 80% yield (Scheme 3). Cbz protection and methylation then gave diester **17** in 69% yield over 2 steps, and Pd^0-catalyzed deallylation furnished the desired D-Glu building block **18** in quantitative yield. For the peptide coupling of **14** with **18**, PyBOP and EDC/HOBt were used to activate **18**. With PyBOP, separation of the product **19** from the phosphoric acid triamide byproduct proved to be difficult, while EDC/HOBt readily afforded **19** in 72% yield. After hydrogenolysis of **19**, the Cbz-deprotected congener **20**, that is, the tetrapeptide building block for the [4+1]-strategy, was obtained in 92% yield (Scheme 3).

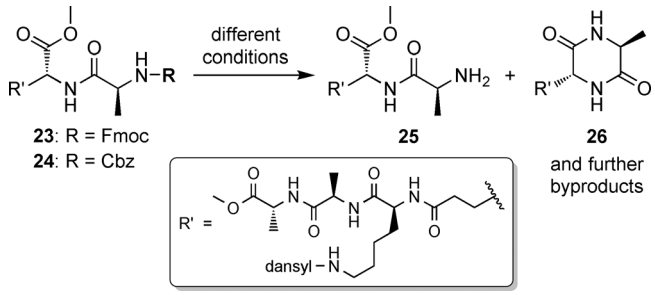
Extension of the tetra- to the pentapeptide was achieved by peptide coupling of **20** with either *N*-Fmoc-L-alanine **21** or *N*-Cbz-L-alanine **22** and EDC/HOBt activation. This led to protected pentapeptides **23** and **24** in yields of 55 and 59%, respectively (Scheme 3). In order to obtain the pentapeptide building block **25** for the [5+0]-strategy, N-terminal deprotection of either **23** or **24** was required (Scheme 4). However, these deprotection reactions proved to be unexpectedly difficult. Fmoc deprotection of **23** with piperidine in DMF mainly led to diketopiperazine formation (byproduct **26**) by intramolecular reaction of the N-terminal amino group with the glutamate methyl ester moiety. We therefore focused our attention on the Cbz deprotection of **24**. In pronounced contrast to the synthesis of dipeptide **11** (vide supra), hydrogenolysis of **24** in the presence of TFA did not fully suppress diketopiperazine formation (Scheme 4). Further byproducts resulted from the unexpected partial hydrolysis of the methyl ester moieties under these conditions, thus leading to cumbersome product mixtures. With respect to these unexpected hurdles, it was decided to discard the [5+0]-strategy and to focus on the [4+1]-route instead.

With key intermediates **7** and **20** for the [4+1]-route in hand, we completed the total synthesis of target compound **3** (Scheme 5). Cleavage of the phenylsulfonylethyl ester moiety of **7** under non-nucleophilic conditions gave carboxylic acid **27**. For the subsequent peptide coupling with **20**, several reagents were studied. Best results were obtained by using HATU, which furnished the desired protected Mur*N*Ac-phosphate pentapeptide **28** in 73% yield (over 2 steps from **7**) without detectable epimerization of the activated L-Ala moiety. The next step was the hydrogenolytic debenzylation of the phosphate moiety. Hitchcock's protocol[14b] with hydrogenation in methanol in the presence of cyclohexylamine did not work in our hands as it led to global basic deprotection of the material. We therefore developed an optimized method for this transformation. Best results were achieved with hydrogenolysis in dioxane in the presence of acetic acid and isolation of the phosphate **29** as its monopyridinium salt. Under these conditions, quantitative yields were feasible, with the phosphate being directly used in the next step without purification.

The formation of the UDP moiety from intermediate **29** was achieved using the morpholidate method established by Khorana and Moffatt,[17a] with Wong's modification to employ 1*H*-tetrazole for activation of the morpholidate.[14c] Thus, **29** was treated with the commercially available UMP-morpholidate **30** in the presence of 1*H*-tetrazole. Subsequent global basic de-



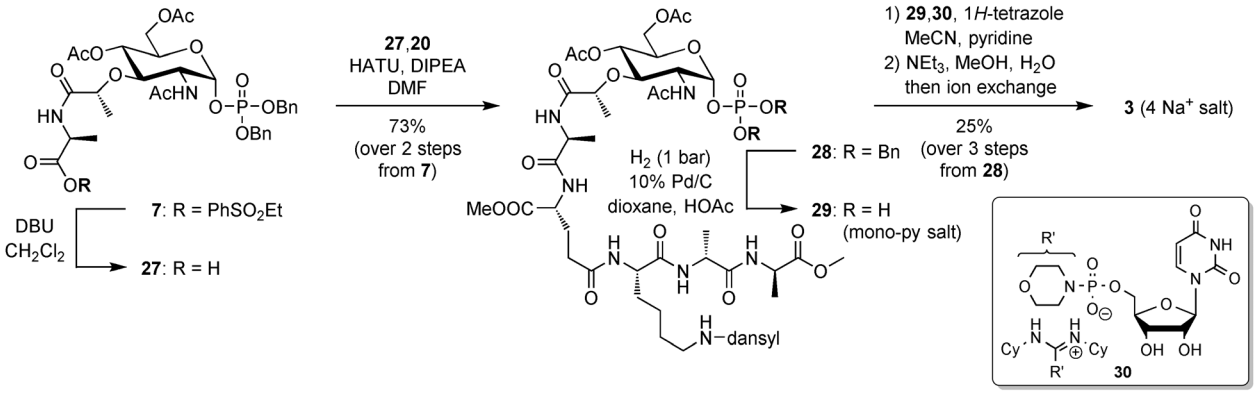
Scheme 3. Synthesis of the tetrapeptide and pentapeptide units **20**, **23** and **24**. PyBOP = (benzotriazol-1-yloxy)-tripyrrolidinophosphonium hexafluorophosphate.



Scheme 4. Attempted deprotection of pentapeptide units **23** and **24**.

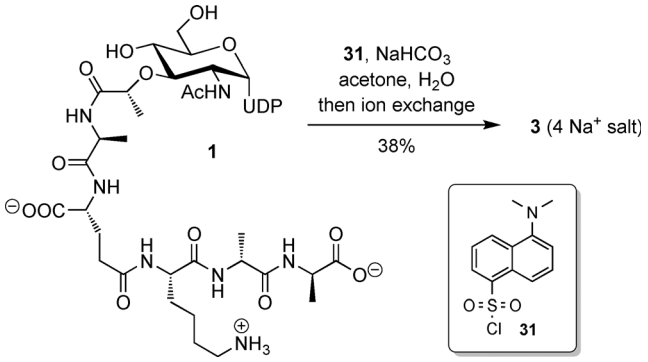
protection, HPLC purification and cation exchange finally furnished target compound **3** (as its tetrasodium salt) in 25% yield over 3 steps from **28** (Scheme 5). We also tested Meier's *cyclo*Sal method as an alternative, robust option for the synthesis of NDP sugars,[17b,c] but in this particular case, the results were less convincing than with the morpholidate approach.

Overall, we have therefore established an efficient total synthesis of dansylated Park's nucleotide **3**, which furnished 65 mg of the target compound, sufficient for numerous MraY assays under high-throughput conditions. The alternative, previously reported approach to obtain **3** is based on the isolation of Park's nucleotide **1** from bacterial cell culture, followed by its dansylation in a semi-synthetic manner.[12c] In order to com-



Scheme 5. Endgame for the synthesis of dansylated Park's nucleotide **3**. Cy = cyclohexyl, HATU = 2-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.

pare both approaches, we followed this established protocol by isolating **1** from bacteria, which was then treated with dansyl chloride **31** in the presence of base (Scheme 6). This was followed by HPLC purification and cation exchange to give **3** (as its tetrasodium salt) in 38% yield. As the amount of **1** isolated from bacterial culture on a conventional laboratory scale was limited, the dansylation could only be carried out on a small scale (e.g., dansylation of 2.5 mg of **1**, furnishing ≈1 mg of the target compound). This semi-synthetic material was identical to the compound obtained by total synthesis as proven by comparison of the ^{1}H NMR spectra and by HPLC co-injection.



Scheme 6. Semi-synthesis of **3** by dansylation of Park's nucleotide **1**.

Finally, both synthetically and semi-synthetically obtained **3** were tested for their substrate properties with recombinantly expressed MraY. We therefore cloned the synthetic *mraY* gene from *Staphylococcus aureus*[7a] into a pet28a vector and transformed *Escherichia coli* Lemo21(DE3) cells for the overexpression of MraY. After cell lysis, MraY assays were performed with the disrupted membranes.[9c,10]

The MraY-catalyzed reaction was monitored by recording the fluorescence over time,[12] with both synthetically and semi-synthetically obtained **3** giving a highly similar increase of fluorescence as anticipated (Figure 2). This increase of fluorescence completely vanished in negative controls using membranes from non-transformed *E. coli* cells. Furthermore, the initially observed increase was significantly reduced in the presence of the commercially available uridine-derived MraY inhibitor tunicamycin.[8] These control experiments confirmed that the increase of fluorescence was specifically correlated to MraY activity and not an artifact resulting from the presence of other membrane components.

Conclusions

In summary, we have completed the first total chemical synthesis of dansylated Park's nucleotide **3**, a substrate analogue of the bacterial membrane protein MraY for fluorescence-based high-throughput MraY assays. We have compared two synthetic strategies towards **3**, with the coupling of a Mur*N*Ac-L-Ala derivative with a tetrapeptide unit ("[4+1]-route") giving the best results. Thus, an unprecedented amount of the target

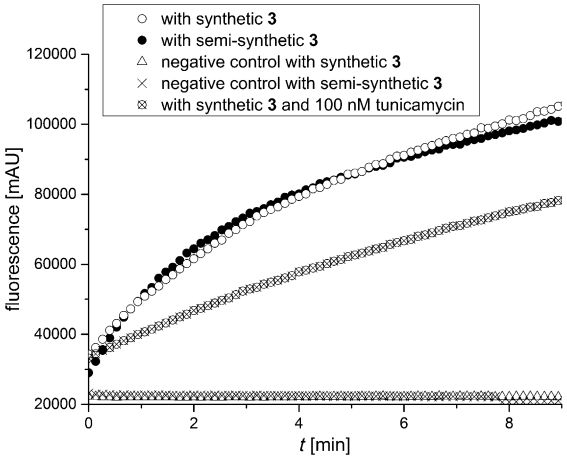


Figure 2. MraY assay with synthetically and semi-synthetically obtained dansylated Park's nucleotide **3** (fluorescence monitoring). Negative controls were obtained by using membrane preparations from non-transformed *E. coli* cells.

compound **3** (65 mg) was obtained, while isolation of Park's nucleotide from bacteria and subsequent dansylation only furnished limited amounts of the MraY substrate analogue. Our work will therefore enable the high-throughput screening of large compound libraries for novel MraY inhibitors. Furthermore, the reported robust total synthesis of dansylated Park's nucleotide **3** will constitute the basis for the preparation of further analogues of the native bacterial metabolite **1**. These analogues will provide unprecedented insights into the substrate specificity and the molecular mechanism of MraY.

Experimental Section

Total synthesis of dansylated Park's nucleotide 3

General methods and the syntheses of all precursor compounds leading to glycosyl phosphate **28** are described in the Supporting Information.

To a solution of protected dansylated 1-(dibenzylphospho)muramic acid pentapeptide **28** (108 mg, 80.0 μmol) in dry dioxane (10 mL), acetic acid (1.0 mL) and Pd (10% on charcoal, 137 mg, 0.130 mmol Pd) were added. The resultant mixture was stirred under an H_2 atmosphere (1 bar) at RT for 22 h. Pyridine (3 mL) was then added and the suspension was stirred at RT for 5 min and then centrifuged. The supernatant was evaporated under reduced pressure, dissolved in water and lyophilized to give slightly impure **29** as a greenish oil (108 mg, 100% yield: 96 mg). The protected dansylated 1-(phospho)muramic acid pentapeptide monopyridinium salt **29** (228 mg, slightly impure material from two debenzylation reactions of **28**) and uridine-5′-monophosphomorpholidate 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salt **30** (182 mg, 0.264 mmol) were co-evaporated separately with dry pyridine (3×5 mL) and then dissolved together in dry pyridine (5 mL). 1*H*-Tetrazole (0.45 M in MeCN, 1.14 mL, 0.510 mmol) was added and the reaction mixture was stirred at RT for 3 d. Water (2 mL) was added and the solvent was evaporated under reduced pressure. The resultant residue was dissolved in a mixture of MeOH, water and NEt_3 (7:3:1, 55 mL) and stirred at RT for 4 d. MeOH was evaporated under reduced pressure and the remaining solution was lyophilized. The re-

sultant crude product was purified by preparative RP-HPLC (see the Supporting Information for details) and converted into the tetrasodium salt by ion exchange chromatography (Dowex 50WX 50–100) to give **3** as a greenish solid (65 mg, 25% over 3 steps from **28**). $[\alpha]_D^{20} = +22.4$ ($c = 1.6$, MeOH). ^{1}H NMR (300 MHz, D_2O): $\delta = 8.48$ (d, $J = 8.7$ Hz, 1H, dansyl-H-2), 8.27 (d, $J = 8.7$ Hz, 1H, dansyl-H-8), 8.23 (d, $J = 7.4$ Hz, 1H, dansyl-H-4), 7.90 (d, $J = 8.1$ Hz, 1H, uracil-H-6), 7.70–7.65 (m, 2H, dansyl-H-3, dansyl-H-7), 7.38 (d, $J = 7.6$ Hz, 1H, dansyl-H-6), 5.95–5.91 (m, 2H, ribose-H-1, uracil-H-5), 5.46 (dd, $J =$ 7.2, 3.2 Hz, 1H, Glc-H-1), 4.34–4.32 (m, 2H, ribose-H-2, Glc-H-4), 4.29–4.23 (m, 4H, Ala-H-2, Glc-H-3, ribose-H-4, propionyl-H-2), 4.23–4.10 (m, 5H, Lys-H-2, Glu-H-2, Glc-H-2, ribose-H-5), 4.10–4.06 (m, 1H, Ala-H-2), 3.96–3.93 (m, 1H, Glc-H-6_a), 3.85–3.59 (m, 6H, 2 x Ala-H-2, Glc-H-4, Glc-H-5, Glc-H-6_b, ribose-H-3), 2.90 (t, $J = 6.5$ Hz, 2H, Lys-H-6), 2.85 (s, 6H, $N(CH_3)_2$), 2.27–2.21 (m, 2H, Glu-H-4), 2.14–2.05 (m, 1H, Glu-H-3_a), 1.99 (s, 3H, *N*-Ac-CH_3), 1.88–1.81 (m, 1H, Glu-H-3_b), 1.41 (d, $J = 7.3$ Hz, 3H, CH_3), 1.38 (d, $J = 6.8$ Hz, 3H, CH_3), 1.42–1.39 (m, 2H, Lys-H-4), 1.30 (d, $J = 7.2$ Hz, 6H, 2 x CH_3), 1.26–1.15 (m, 2H, Lys-H-5), 1.13–1.03 (m, 1H, Lys-H-3_a), 1.03–0.94 ppm (m, 1H, Lys-H-3_b); ^{13}C NMR (75 MHz, D_2O): $\delta = 179.8$ (C=O), 177.6 (C=O), 175.8 (C=O), 175.4 (C=O), 174.2 (C=O), 174.0 (C=O), 173.5 (C=O), 152.2 (uracil-C-4), 150.9 (uracil-C-2, dansyl-C-5), 141.6 (uracil-C-6), 134.1 (dansyl-C-1), 130.2 (dansyl-C-2), 129.8 (dansyl-C-4), 129.0 (dansyl-C-8a), 129.0 (dansyl-C-4a), 128.8 (dansyl-C-7), 124.1 (dansyl-C-3), 119.1 (dansyl-C-8), 116.0 (dansyl-C-6), 102.7 (uracil-C-5), 94.7 (d, $J_{CP} = 5.5$ Hz, Glc-C-1), 88.6 (ribose-C-1), 83.2 (d, $J_{CP} = 9.2$ Hz, ribose-C-4), 79.9 (propionyl-C-2), 78.0 (Glc-C-3), 73.8 (ribose-C-2), 73.0 (ribose-C-3), 69.7 (Glc-C-4), 68.1 (Glc-C-5), 65.1 (d, $J_{CP} = 4.2$ Hz, ribose-C-5), 60.4 (Glc-C-6), 54.4 (Ala-C-2), 54.2 (Ala-C-2), 53.5 (d, $J_{CP} = 8.2$ Hz, Glc-C-2), 51.0 (Ala-C-2), 49.8 (Glu-C-2), 52.0 (Lys-C-2), 45.0 ($N(CH_3)_2$), 42.2 (Lys-C-6), 31.9 (Glu-C-4), 30.3 (Lys-C-4), 28.2 (Glu-C-3), 27.8 (Lys-C-5), 22.2 (*N*-Ac-CH_3), 22.0 (Lys-C-3), 18.8 (CH_3), 17.6 (CH_3), 17.0 (CH_3), 16.6 ppm (CH_3); ^{31}P NMR (121 MHz, D_2O): $\delta = -11.11$ (d, $J = 20.6$ Hz, 1P), -12.92 ppm (d, $J = 20.6$ Hz, 1P); IR (ATR): $\tilde{\nu} = 3294$, 2943, 1741, 1695, 1634, 1541, 1455, 1145, 791, 628 cm^{-1}. HRMS (ESI): calcd for $C_{52}H_{75}N_{10}O_{28}P_2S$ 1381.3954, found 1381.3944 $[M-H]^-$.

Semi-synthesis of dansylated Park's nucleotide 3

The isolation of naturally occurring Park's nucleotide **1** was carried out according to a modified protocol from Kohlrausch and Hölt-je.[18a] *S. aureus* SA113 was grown in Antibiotic Medium 3 (Difco, 3× 100 mL) at 37 °C and 140 rpm for 24 h as precultures ($OD_{578} = 1.1$–1.2). From each preculture, approximately 70 mL were transferred into Antibiotic Medium 3 (2 L each, starting $OD_{578} \approx 0.06$) to give the main cultures. These were incubated at 37 °C and 140 rpm until OD_{578} 0.8–0.9 was reached. Penicillin G (Sigma Aldrich, 75 μg mL^{-1} cell culture) was added and the mixtures were incubated at 37 °C for 1 h under stirring. Cells were collected by centrifugation (12000 *g*, 4 °C, 15 min, at this point cells from all cultures were combined), resuspended in water (0.1 g wet weight mL^{-1} water) and slowly stirred into boiling water (double volume). Boiling was continued for 30 min, the suspension was allowed to cool to RT and finally stirred at 4 °C overnight. It was then centrifuged (200000 *g*, 4 °C, 1 h) and the supernatant was lyophilized. The resultant residue was dissolved in water and applied to gel permeation chromatography (GPC, column Toyopearl TSK-40F (Tosoh Bioscience, 2.5×120 cm), eluent 8:20:2000 pyridine-AcOH-water) to yield crude **1**. Further purification was achieved by preparative RP-HPLC and final desalting by GPC as described before for UDP-muramyl tripeptide (DAP-type)[18b] to give **1** (≈10 mg from the 6 L of bacterial culture).

To a solution of the thus obtained Park's nucleotide **1** (2.5 mg, 2.2 μmol) in acetone (190 μL), dansyl chloride (5.9 mg, 22 μmol) and $NaHCO_3$ (0.25 M in water, 190 μL, 48 μmol) were added. The reaction mixture was stirred at 24 °C for 3 h. After centrifugation, resuspension of the pellet in water (270 μL) and repeated centrifugation, the supernatants were combined and evaporated under reduced pressure. The resultant residue was resuspended in water (500 μL) and centrifuged again. The supernatant was purified by semi-preparative RP-HPLC (see the Supporting Information for details) and converted into the tetrasodium salt by ion exchange chromatography (Dowex 50WX 50–100) to give **3** as a slightly greenish solid (1.2 mg, 38%). ^{1}H NMR spectra and MS data of this material were identical to the corresponding data for synthetically obtained **3**.

Cloning of the *mraY* gene from *S. aureus*

The *mraY* gene of *S. aureus* subsp. *aureus* MRSA252 (BX571856.1) was purchased from Mr. Gene (see the Supporting Information for the gene sequence) and provided in a pMA vector. This *mraY* gene was cut with the restriction enzymes NcoI and XhoI (New England Biolabs) and ligated into a pET28a-(+) vector (Novagen), which had been cut by using the same enzymes. The resultant plasmid thus featured a gene encoding the MraY enzyme with a C-terminal His_6 tag. The plasmid was amplified with the *E. coli* strain XL-1 blue based on the kanamycin resistance provided by the pET28a vector, and its sequence was confirmed by DNA sequencing (Eurofins MWG Operon).

Overexpression of MraY from *S. aureus* in *E. coli*

The *mraY*-containing plasmid was transformed into *E. coli* Lemo21 cells (New England Biolabs), which were plated on lysogeny broth (LB) agar containing kanamycin (50 μg mL^{-1}) and chloramphenicol (30 μg mL^{-1}). A single colony was picked to induce an overnight culture (10 mL) of LB media containing kanamycin (50 μg mL^{-1}) and chloramphenicol (30 μg mL^{-1}), which was incubated at 37 °C and 180 rpm for 16 h. A culture of LB media (500 mL) containing kanamycin (50 μg mL^{-1}), chloramphenicol (30 μg mL^{-1}) and L-rhamnose (1 mM) was inoculated with the overnight culture (500 μL) and then grown at 37 °C and 180 rpm to OD_{600} 0.6. This was followed by induction of MraY expression with IPTG (1 mM) and incubation at 37 °C and 180 rpm for 4 h. Cells were centrifuged (4600 *g*, 20 min, 4 °C) and the pellet was resuspended in buffer A (50 mM Tris-HCl buffer pH 7.5, 1 mM $MgCl_2$, 2 mM β-mercaptoethanol; 15 mL total). Egg white lysozyme (spatula tip), DNAse I (spatula tip) and a tablet of cOmplete™ EDTA-free protease inhibitor cocktail (Merck) were added. Cells were lysed by using sonication (30%, 30/100 pulse, 15 min on ice) and then incubated at 4 °C for 30 min. The lysate was centrifuged (17000 *g*, 45 min, 4 °C) and the supernatant was centrifuged again (180000 *g*, 1 h, 4 °C). The resultant pellet was resuspended in buffer A (1.7 mL), flash frozen in liquid nitrogen and stored at −80 °C (aliquots of 20 μL). This MraY-containing crude membrane preparation (overall protein concentration ≈20.5 mg mL^{-1} as determined by OD_{280}, Nanodrop UV spectrometer) was diluted with water (final overall protein concentration 1 mg mL^{-1}) and then directly used for MraY activity assays without further purification.[9c,10]

Fluorescence-based MraY assay

Based on Bugg's method,[9c,10,12] fluorescence intensity over time was measured at $\lambda_{ex} = 355$ nm and $\lambda_{em} = 520$ nm (BMG Labtech POLARstar Omega, 384-well plate format). To start the MraY-catalyzed

reaction, the crude membrane preparation of MraY from *S. aureus* (1 μL) was added to a mixture of undecaprenyl phosphate (50 μM) and dansylated Park's nucleotide **3** (synthetic or semi-synthetic, 7.5 μM) in buffer (100 mM Tris-HCl buffer pH 7.5, 200 mM KCl, 10 mM $MgCl_2$, 0.1% Triton X-100, 20 μL overall). Negative controls were obtained using membrane preparations from non-transfected *E. coli* Lemo21 cells. To demonstrate the partial inhibition of MraY activity by an established MraY inhibitor, tunicamycin (Sigma–Aldrich) was added to the reaction mixture at 100 nM concentration.

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft (DFG, SFB 803 "Functionality controlled by organization in and between membranes" and EXC 306 "Inflammation at Interfaces") and the Fonds der Chemischen Industrie (FCI, Sachkostenzuschuss) for financial support. Technical assistance of B. Buske (Research Center Borstel) is gratefully acknowledged.

Keywords: antibiotics · enzyme assays · fluorescence · medicinal chemistry · peptidoglycan

[1] a) G. Taubes, *Science* **2008**, *321*, 356 – 361; b) M. A. Cooper, D. Shlaes, *Nature* **2011**, *472*, 32.

[2] a) F. von Nussbaum, M. Brands, B. Hinzen, S. Weigand, D. Häbich, *Angew. Chem. Int. Ed.* **2006**, *45*, 5072 – 5129; *Angew. Chem.* **2006**, *118*, 5194 – 5254; b) R. B. Hamed, J. R. Gomez-Castellanos, L. Henry, C. Ducho, M. A. McDonough, C. J. Schofield, *Nat. Prod. Rep.* **2013**, *30*, 21 – 107.

[3] a) C. Dini, *Curr. Top. Med. Chem.* **2005**, *5*, 1221 – 1236; b) T. D. H. Bugg, A. J. Lloyd, D. I. Roper, *Infect. Disord.: Drug Targets* **2006**, *6*, 85 – 106.

[4] a) W. G. Struve, F. C. Neuhaus, *Biochem. Biophys. Res. Commun.* **1965**, *18*, 6 – 12; b) J. S. Anderson, M. Matsuhashi, M. A. Haskin, J. L. Strominger, *Proc. Natl. Acad. Sci. USA* **1965**, *53*, 881 – 889; c) M. G. Heydanek Jr., W. G. Struve, F. C. Neuhaus, *Biochemistry* **1969**, *8*, 1214 – 1221; d) M. Ikeda, M. Wachi, H. K. Jung, F. Ishino, M. Matsuhashi, *J. Bacteriol.* **1991**, *173*, 1021 – 1026; e) D. S. Boyle, W. D. Donachie, *J. Bacteriol.* **1998**, *180*, 6429 – 6432.

[5] a) A. Bouhss, D. Mengin-Lecreulx, D. Le Beller, J. van Heijenoort, *Mol. Microbiol.* **1999**, *34*, 576 – 585; b) B. C. Chung, J. Zhao, R. A. Gillespie, D.-Y. Kwon, Z. Guan, J. Hong, P. Zhou, S.-Y. Lee, *Science* **2013**, *341*, 1012 – 1016.

[6] a) A. J. Lloyd, P. E. Brandish, A. M. Gilbey, T. D. H. Bugg, *J. Bacteriol.* **2004**, *186*, 1747 – 1757; b) B. Al-Dabbagh, X. Henry, M. E. Ghachi, G. Auger, D. Blanot, C. Parquet, D. Mengin-Lecreulx, A. Bouhss, *Biochemistry* **2008**, *47*, 8919 – 8928; c) B. Al-Dabbagh, S. Olatunji, M. Crouvoisier, M. El Ghachi, D. Blanot, D. Mengin-Lecreulx, A. Bouhss, *Biochimie* **2016**, *127*, 249 – 257.

[7] a) A. Bouhss, M. Crouvoisier, D. Blanot, D. Mengin-Lecreulx, *J. Biol. Chem.* **2004**, *279*, 29974 – 29980; b) Y. Ma, D. Münch, T. Schneider, H.-G. Sahl, A. Bouhss, U. Ghoshdastider, J. Wang, V. Dötsch, X. Wang, F. Bernhard, *J. Biol. Chem.* **2011**, *286*, 38844 – 38853; c) E. Henrich, Y. Ma, I. Engels, D. Münch, C. Otten, T. Schneider, B. Henrichfreise, H.-G. Sahl, V. Dötsch, F. Bernhard, *J. Biol. Chem.* **2016**, *291*, 2535 – 2546.

[8] a) K.-I. Kimura, T. D. H. Bugg, *Nat. Prod. Rep.* **2003**, *20*, 252 – 273; b) M. Winn, R. J. M. Goss, K.-I. Kimura, T. D. H. Bugg, *Nat. Prod. Rep.* **2010**, *27*, 279 – 304; c) S. Ichikawa, M. Yamaguchi, A. Matsuda, *Curr. Med. Chem.* **2015**, *22*, 3951 – 3979; d) D. Wiegmann, S. Koppermann, M. Wirth, G. Niro, K. Leyerer, C. Ducho, *Beilstein J. Org. Chem.* **2016**, *12*, 769 – 795; e) T. D. H. Bugg, M. T. Rodolis, A. Mihalyi, S. Jamshidi, *Bioorg. Med. Chem.* **2016**, DOI: 10.1016/j.bmc.2016.03.018.

[9] a) T. Tanino, B. Al-Dabbagh, D. Mengin-Lecreulx, A. Bouhss, H. Oyama, S. Ichikawa, A. Matsuda, *J. Med. Chem.* **2011**, *54*, 8421 – 8439; b) Y. Takeoka, T. Tanino, M. Sekiguchi, S. Yonezawa, M. Sakagami, F. Takahashi, H. Togame, Y. Tanaka, H. Takemoto, S. Ichikawa, A. Matsuda, *ACS Med. Chem. Lett.* **2014**, *5*, 556 – 560; c) A. P. Spork, M. Büschleb, O. Ries, D. Wiegmann, S. Boettcher, A. Mihalyi, T. D. H. Bugg, C. Ducho, *Chem. Eur. J.* **2014**, *20*, 15292 – 15297.

[10] a) M. T. Rodolis, A. Mihalyi, A. O'Reilly, J. Slikas, D. I. Roper, R. E. W. Hancock, T. D. H. Bugg, *ChemBioChem* **2014**, *15*, 1300 – 1308; b) M. T. Rodolisa, A. Mihalyi, C. Ducho, K. Eitel, B. Gust, R. J. M. Goss, T. D. H. Bugg, *Chem. Commun.* **2014**, *50*, 13023 – 13025.

[11] B. C. Chung, E. H. Mashalidis, T. Tanino, M. Kim, A. Matsuda, J. Hong, S. Ichikawa, S.-Y. Lee, *Nature* **2016**, *533*, 557 – 560.

[12] a) P. E. Brandish, M. K. Burnham, J. T. Lonsdale, R. Southgate, M. Inukai, T. D. H. Bugg, *J. Biol. Chem.* **1996**, *271*, 7609 – 7614; b) P. E. Brandish, K.-I. Kimura, M. Inukai, R. Southgate, J. T. Lonsdale, T. D. H. Bugg, *Antimicrob. Agents Chemother.* **1996**, *40*, 1640 – 1644; c) T. Stachyra, C. Dini, P. Ferrari, A. Bouhss, J. van Heijenoort, D. Mengin-Lecreulx, D. Blanot, J. Biton, D. Le Beller, *Antimicrob. Agents Chemother.* **2004**, *48*, 897 – 902.

[13] A. B. Shapiro, H. Jahić, N. Gao, L. Hajec, O. Rivin, *J. Biomol. Screening* **2012**, *17*, 662 – 672.

[14] a) R. S. Narayan, M. S. VanNieuwenhze, *Eur. J. Org. Chem.* **2007**, 1399 – 1414; b) S. A. Hitchcock, C. N. Eid, J. A. Aikins, M. Zia-Ebrahimi, L. C. Blaszczak, *J. Am. Chem. Soc.* **1998**, *120*, 1916 – 1917; c) H. Liu, R. Sadamoto, P. S. Sears, C.-H. Wong, *J. Am. Chem. Soc.* **2001**, *123*, 9916 – 9917; d) M. Kurosu, S. Mahapatra, P. Narayanasamy, D. C. Crick, *Tetrahedron Lett.* **2007**, *48*, 799 – 803; e) K. Li, M. Kurosu, *Heterocycles* **2008**, *76*, 455 – 469; f) K.-T. Chen, Y.-C. Kuan, W.-C. Fu, P.-H. Liang, T.-J. R. Cheng, C.-H. Wong, W.-C. Cheng, *Chem. Eur. J.* **2013**, *19*, 834 – 838; g) L.-Y. Huang, S.-H. Huang, Y.-C. Chang, W.-C. Cheng, T.-J. R. Cheng, C.-H. Wong, *Angew. Chem. Int. Ed.* **2014**, *53*, 8060 – 8065; *Angew. Chem.* **2014**, *126*, 8198 – 8203.

[15] A. D. de Araujo, J. M. Palomo, J. Cramer, O. Seitz, K. Alexandrov, H. Waldmann, *Chem. Eur. J.* **2006**, *12*, 6095 – 6109.

[16] a) T. H. Chan, M. A. Brook, *Synthesis* **1983**, 201 – 203; b) K. L. Webster, A. B. Maude, M. E. O'Donnell, A. P. Mehrotra, D. Gani, *J. Chem. Soc. Perkin Trans. 1* **2001**, 1673 – 1695.

[17] a) S. Roseman, J. J. Distler, J. G. Moffatt, H. G. Khorana, *J. Am. Chem. Soc.* **1961**, *83*, 659 – 663; b) S. Wendicke, S. Warnecke, C. Meier, *Angew. Chem. Int. Ed.* **2008**, *47*, 1500 – 1502; *Angew. Chem.* **2008**, *120*, 1523 – 1525; c) S. Wolf, T. Zismann, N. Lunau, C. Meier, *Chem. Eur. J.* **2009**, *15*, 7656 – 7664.

[18] a) U. Kohlrausch, J.-V. Höltje, *FEMS Microbiol. Lett.* **1991**, *78*, 253 – 258; b) N. Gisch, B. Buske, H. Heine, B. Lindner, U. Zähringer, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3362 – 3366.

Received: September 9, 2016
Published online on ■■ ■■, 0000

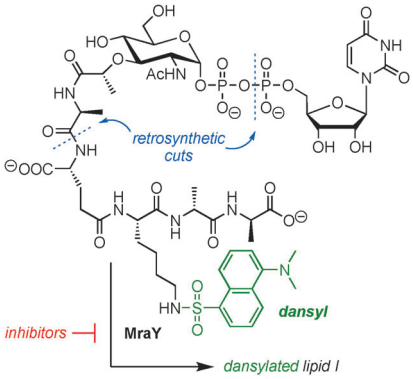
FULL PAPER

Medicinal Chemistry

*S. Wohnig, A. P. Spork, S. Koppermann, G. Mieskes, N. Gisch, R. Jahn, C. Ducho**

■■ – ■■

Total Synthesis of Dansylated Park's Nucleotide for High-Throughput MraY Assays



Total, not semi: Park's nucleotide is an intermediate in bacterial peptidoglycan biosynthesis acting as the substrate of the enzyme MraY. An efficient total synthesis of dansylated Park's nucleotide is reported. The thus obtained amount of the target compound vastly exceeded the amount accessible by semi-synthesis on conventional scale, therefore enabling high-throughput assays for the identification of MraY inhibitors as potential antimicrobial agents.