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Synthesis, Antimicrobial Activity, and Membrane Permeabilizing Properties of C-Terminally Modified Nisin Conjugates Accessed by CuAAC

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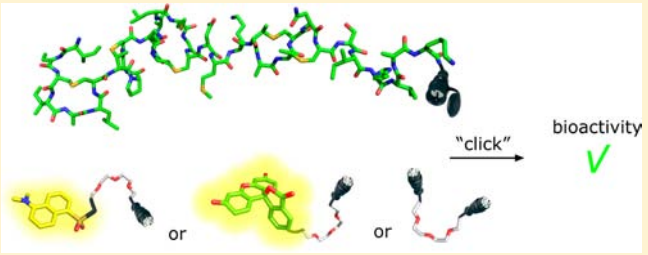
Supporting Information

ABSTRACT: Functionalization of the lantibiotic nisin with fluorescent reporter molecules is highly important for the understanding of its mode of action as a potent antimicrobial peptide. In addition to this, multimerization of nisin to obtain multivalent peptide constructs and conjugation of nisin to bioactive molecules or grafting it on surfaces can be attractive methods for interference with bacterial growth. Here, we report a convenient method for the synthesis of such nisin conjugates and show that these nisin derivatives retain both their antimicrobial activity and their membrane permeabilizing properties. The synthesis is based on the Cu(I)-catalyzed alkyne−azide cycloaddition reaction (CuAAC) as a bioorthogonal ligation method for large and unprotected peptides in which nisin was C-terminally modified with propargylamine and subsequently efficiently conjugated to a series of functionalized azides. Two fluorescently labeled nisin conjugates together with a dimeric nisin construct were prepared while membrane insertion as well as antimicrobial activity were unaffected by these modifications. This study shows that C-terminal modification of nisin does not deteriorate biological activity in sharp contrast to N-terminal modification and therefore C-terminally modified nisin analogues are valuable tools to study the antibacterial mode of action of nisin. Furthermore, the ability to use stoichiometric amounts of the azide containing molecule opens up possibilities for surface tethering and more complex multivalent structures.



INTRODUCTION

The antimicrobial peptide nisin belongs to the class of lantibiotics,[1−3] a potent natural subset of antibiotic molecules characterized by specifically modified amino acids. Lantibiotics are ribosomally synthesized peptides and undergo several post-translational modifications induced by a variety of enzymes, comprising regiospecific dehydration of serine and threonine residues and subsequent stereoselective intramolecular cyclization of these dehydroamino acids with cysteine residues.[4,5] These post-translational modifications lead to the unique structural characteristics of lantibiotics, since the presence of dehydrated amino acids in combination with lanthionines (thioether bridges) give these peptides a rigid conformation which is of utmost importance for their antimicrobial activity. Nisin consists of 34 amino acid residues and has an elongated structure with an overall positive charge and contains five cyclic constrained A-, B-, C-, as well as the knotted DE-ring systems (Figure 1). Nisin is a very potent antimicrobial peptide, active at nanomolar concentrations against a broad spectrum of Gram-positive bacteria, for example, as a food additive (E234) and, among others, in antimicrobial materials and coatings.[6−8] Nisin has a 2-fold mode of action. First, it inhibits bacterial cell-wall synthesis, by binding of the N-terminal AB-ring fragment to lipid II, an essential precursor cross-linking molecule during the bacterial cell-wall synthesis. Second, after binding to lipid II, the C-terminus inserts into the phospholipid membrane forming a pore-complex, which leads to a collapse of vital ion gradients and ultimately results in bacterial cell death.[9−13] Although the molecular interaction of nisin and lipid II is well described, details of nisin-induced pore formation still remain elusive, and to study this process, fluorescently labeled nisin derivatives are important tools for the chemical biologist.

Selective functionalization of nisin is rather difficult due to the presence of several reactive side chain functionalities. Although the total synthesis of nisin[14] and syntheses of nisin analogues[15−19] have been published, it is not a realistic

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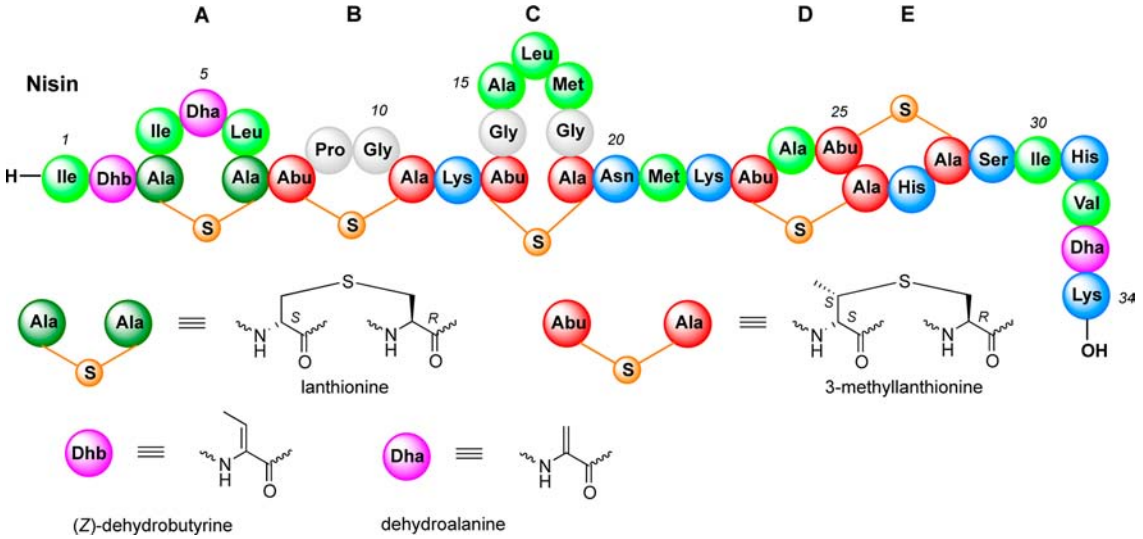

Figure 1. Schematic representation of the structure of nisin. Hydrophobic amino acids are shown in light green, polar residues in blue, unsaturated amino acid residues in purple, while the lanthionine is represented in dark green and the 3-methyllanthionines in red. The structural formulas of lanthionine, 3-methyllanthionine, dehydroalanine, and (*Z*)-dehydrobutyrine are also given.

approach to design a unique synthesis route for each single modified analogue. Since nisin contains four primary amines, the N-terminal α-amino group and three ε-amines of Lys12, 22, and 34, respectively (Figure 1), the regioselective modification of these amine functionalities is, however, far from trivial. Nevertheless, the selective N-terminal α-amine modification has been successfully performed making use of differences in pK_a-values of these amines by careful pH control.[20] However, a crucial feature of the antimicrobial activity of nisin resides in the binding of the N-terminal AB-ring system to lipid II and any modification of the N-terminus will interfere with this critical interaction, resulting in a significantly reduced bioactivity. Indeed, Veronese and co-workers showed that PEG-ylation of the N-terminal α-amine functionality of nisin resulted in a complete loss of antimicrobial activity.[21] Another functional moiety within nisin as a conjugation target is bestowed by the dehydrated residues (Dhb2, Dha5, and Dha33; see Figure 1), in which the Cα-Cβ double bond in combination with the carbonyl of the amide bond forms a typical Michael acceptor and can be used in chemoselective conjugation reactions, like thiol−ene 'click' approaches.[22] Within this context, Liu and Hansen showed that, although nisin rapidly reacted with thiols, presumably via these Michael acceptors, the resulting conjugate did not show any antimicrobial activity.[23]

Since nisin contains only a single carboxylic acid moiety as part of the C-terminus, this functionality can be conveniently used for the regioselective modification of the antimicrobial peptide, and several examples have been reported in the literature in which fluorescent molecules,[9,24,25] biotin labels,[26] and amino-PEG chains[21] have been used. Modification of this carboxylic acid functionality by amide bond formation is not always ideal, since often excess of relatively expensive amine derivatives have to be used. Furthermore, the need to use a large excess of amine makes the synthesis of multivalent constructs or complex conjugates such as surface tethered nisin far from trivial, as the excess of the amine may be difficult to remove from these complex molecular constructs. However, a stoichiometric amount of the amine may not be sufficient for an efficient coupling and side reactions may occur, such as self-condensation. Here, we present a more convenient approach, namely, the C-terminal modification of nisin with a cheap amine of which excess can be easily removed and that after coupling can be used as a bio-orthogonal conjugation handle for chemoselective coupling of the reporter molecule of choice. With respect to this, the Cu(I)-catalyzed alkyne−azide cycloaddition reaction (CuAAC)[27−30] is ideal, since nisin can be conveniently derivatized with propargylamine and the resulting peptide-alkyne can be conjugated in stoichiometric amounts to a variety of functionalized azides.

Thus, we describe the convenient synthesis of a C-terminally functionalized nisin-alkyne derivative and its subsequent application in Cu(I)-catalyzed cycloaddition chemistry with two fluorescent azides and a bis-azide, which afforded two fluorescently labeled nisin derivatives and a dimeric nisin construct, respectively, all in good yields (31−52%). The dimer construct was synthesized to show the potential of this strategy for multimerization of nisin. All nisin conjugates were biologically active as potent inhibitors of bacterial growth and retained their membrane permeabilizing properties.

MATERIALS AND METHODS

General. Unless stated otherwise, all chemicals were obtained from commercial sources and used without further purification. The active ester, 5(6)-carboxyfluorescein-OSu (**12**), was purchased from Thermo Scientific (Breda, The Netherlands). All reactions were performed at ambient temperature and under an air atmosphere, unless stated specifically at each entry. *N*,*N*-Diisopropylethylamine (DIPEA), *N*,*N*-dimethylformamide (DMF), and HPLC grade solvents were purchased from Biosolve B.V. (Valkenswaard, The Netherlands) and used as supplied, with the exception of DMF and CH_2Cl_2, which were dried over molecular sieves (4 Å) prior to use. Progress of reactions was monitored by TLC on Merck precoated silica gel 60F254 glass plates. Spots were visualized by UV quenching and staining with ninhydrin. Column chromatography was performed on Silicycle SiliFlash P60 silica gel (particle size 40−63 μm). ^{1}H NMR spectra were acquired on a Varian Mercury 300 MHz spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to TMS (0.00 ppm). Coupling constants (J) are

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reported in Hertz (Hz). Splitting patterns are designated as singlet (s), doublet (d), triplet (t), multiplet (m), and broad (br). ^{13}C NMR data were acquired on a Varian Mercury 75.5 MHz spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to the solvent residual signal $CDCl_3$ (77.0 ppm). Microwave reactions were performed in a Biotage Initiator microwave reactor, equipped with a temperature and pressure control, in sealed vessels of 0.5−2 mL. Analytical HPLC was performed on a Shimadzu automated HPLC system equipped with a UV/vis detector operating at 220/254 nm. Preparative HPLC runs were performed on an Applied Biosystems 400 solvent delivery system with an Applied Biosystems 757 UV/vis absorbance detector. The buffer system for HPLC consisted of buffer A: 0.1% TFA in MeCN/H_2O 5:95 v/v and buffer B: 0.1% TFA in MeCN/H_2O 95:5 v/v. For analytical HPLC a flow rate of 1.0 mL/min with a linear gradient of buffer A to buffer B (60% in 20 min) was used with a total run time of 40 min using an Alltech C8 Alltima column (pore size: 100 Å, particle size: 5 μm, 250 × 4.6 mm). Preparative runs used a flow rate of 12 mL/min with a linear gradient of buffer B (60% in 60 min) from 100% buffer A with a total runtime of 80 min using an Alltech C18 Prosphere column (pore size: 300 Å, particle size: 10 μm, 250 × 22 mm). Semipreparative HPLC runs were performed at a flow rate of 5 mL/min with a linear gradient of buffer B (60% in 60 min) from 100% buffer A with a total runtime of 80 min using a Grace HP C18 Prosphere column (pore size: 300 Å, particle size: 10 μm, 250 × 10 mm). Peptides were characterized using ElectroSpray Ionization Mass Spectrometry (ESI-MS) on a Shimadzu QP8000 single quadrupole mass spectrometer in a positive ionization mode. Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) MS spectra were recorded on a Shimadzu Axima-CFR with α-cyano-4-hydroxycinnamic acid as the matrix, while human ACTH(18−39) was used as an external reference (monoisotopic $[M+H]^+$ 2465.198).

*Nisin-Alkyne **2**.* Nisin **1**[31] (250 mg, 25 μmol) was suspended in DMF (1000 μL) and to this brown suspension, propargylamine (120 μL, 1.88 mmol, 25 equiv) and BOP (33.2 mg, 75.0 μmol) were added and the obtained reaction mixture was shaken for 20 min. Then, the reaction mixture was neutralized by the dropwise addition of aq. 1 M HCl, and after evaporation of the solvents *in vacuo*, the residue was dissolved in HPLC buffer (2 mL, A/B 3:1 v/v) and subsequently purified by preparative HPLC. After lyophilization of the pure fractions, nisin-alkyne **2** was obtained as a white fluffy powder with 59% yield (50 mg). R_t 23.93 min; MALDI-TOF-MS calcd. for $C_{146}H_{233}N_{43}O_{36}S_7$: 3388.58, found m/z 3390.11 $[M+H]^+$, 3412.06 $[M+Na]^+$.

*O-(3-Aminopropyl)-O′-(N-Boc-aminopropyl)diethylene glycol **4**.* A solution of Boc_2O (3.4 g, 15.6 mmol) in dioxane (100 mL) was added dropwise to a solution of *O*,*O*′-bis(3-aminopropyl)diethylene glycol **3** (24.0 g, 109.2 mmol) in H_2O (100 mL) and the reaction mixture was stirred for 17 h. After removing the solvent *in vacuo*, the residual oil was dissolved in water (150 mL) and filtered over HyFlo to remove the bis-carbamate side-product. The water layer was extracted with EtOAc (3 × 150 mL) and the combined organic layers were dried (Na_2SO_4), filtrated, and concentrated *in vacuo* to give carbamate **4** as a colorless syrup with an excellent yield of 96% (4.8 g, 14.92 mmol). ^1H NMR (300 MHz, D_2O) δ = 3.54 (s, 8H, O−CH_2 (4 × 2H)), 3.47 (t (J = 6.6 Hz), 4H, ∼O−CH_2−CH_2−CH_2∼), 3.01 (t (J = 6.7 Hz), 2H, CH_2-N(H)Boc), 2.55 (t (J = 7.0 Hz), 2H, CH_2-NH_2), 1.61 (m, 4H, ∼O−CH_2−CH_2−CH_2∼), 1.30 (s, 9H, Boc). This ^1H NMR spectrum was in agreement with the reported literature data.[32]

*O-(3-Azidopropyl)-O′-(N-Boc-aminopropyl)diethylene glycol **5**.* Amine **4** (1.75 g, 5.5 mmol) was dissolved in MeOH (50 mL). Then, a solution of $CuSO_4·5H_2O$ (27 mg, 0.11 mmol) and $NaHCO_3$ (1.40 g, 16.4 mmol) in H_2O (25 mL) was subsequently added to the solution of **4** in MeOH and a clear blue reaction mixture was obtained. The diazotransfer reagent imidazole-sulfonyl azide hydrochloride **10**[33] (1.40 g, 6.55 mmol) was added portionwise and the conversion from amine **4** into azide **5** was complete after 2 h of stirring according to TLC analysis. Subsequently, the pale green reaction mixture was concentrated *in vacuo* to approximately 25 mL and the aqueous layer was extracted with EtOAc (3 × 20 mL). The combined organic layers were subsequently washed with aq. 1 M $KHSO_4$ (3 × 40 mL), aq. 5% $NaHCO_3$ (3 × 40 mL), and brine (3 × 40 mL), dried (Na_2SO_4), filtrated, and concentrated under reduced pressure to give azide **5** in high yield (82%) as a colorless oil (1.55 g, 4.5 mmol). R_f (MeOH/$CHCl_3$/AcOH 89:10:1 v/v/v): 0.67; ^1H NMR (300 MHz, $CDCl_3$) δ = 5.02 (s, 1H, N(H)Boc), 3.72−3.49 (m, 12H, O−CH_2 (6 × 2H)), 3.40 (t (J = 6.7 Hz), 2H, CH_2-N_3), 3.22 (m, 2H, CH_2-NHBoc), 1.96−1.81 (m, 2H, ∼CH_2−CH_2−CH_2−N_3), 1.75 (m, 2H, ∼CH_2−CH_2−CH_2−N(H)Boc), 1.44 (s, 9H, Boc); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 156.1, 78.9, 70.5, 70.5, 70.3, 70.2, 69.5, 67.9, 48.4, 38.4, 29.6, 29.0, 28.4.

*O-(3-Azidopropyl)-O′-(aminopropyl)diethylene glycol hydrochloride **6**.* Boc-protected amine **5** (1.55 g, 4.48 mmol) was dissolved in CH_2Cl_2 (40 mL) and a saturated solution of dry HCl in Et_2O (20 mL) was added. Boc-removal was complete after 4 h of stirring and the reaction mixture was subsequently evaporated to dryness. The residue was coevaporated with MeOH (3 × 10 mL) and CH_2Cl_2 (3 × 10 mL) to remove any residual acid and hydrochloride **6** was obtained in quantitative yield (1.26 g). R_f (CH_2Cl_2/MeOH 9:1 v/v): 0.16; ^1H NMR (300 MHz, $CDCl_3$) δ = 8.11 (s, 3H, ∼NH_2·HCl), 3.63−3.52 (m, 8H, O−CH_2 (4 × 2H)), 3.47 (m, 4H, ∼O−CH_2−CH_2−CH_2∼ (2 × 2H)), 3.35 (m, 2H, CH_2-N_3), 3.09 (m, 2H, CH_2-NH_2·HCl), 1.98 (m, 2H, ∼CH_2−CH_2−CH_2−N_3), 1.77 (m, 2H, ∼CH_2−CH_2−CH_2−NH_2·HCl); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 70.7, 70.5, 70.4, 70.2, 69.3, 68.1, 48.6, 39.0, 29.2, 27.1.

*O,O′-Bis(3-azidopropyl)diethylene glycol **7**.* *O*,*O*′-Bis(3-aminopropyl)diethylene glycol (**3**) (1.10 g, 5.0 mmol) was dissolved in MeOH (40 mL) and a solution containing $CuSO_4$ (15 mg, 0.05 mmol) and K_2CO_3 (2.07 g, 15.0 mmol) in H_2O (20 mL) was added resulting in a clear blue reaction mixture. Then, imidazole-sulfonyl azide hydrochloride **10**[33] (2.51 g, 12.0 mmol) was added portionwise and the diazotransfer reaction was complete after 2 h. The greenish reaction mixture was concentrated to approximately 20 mL by evaporation and the residue was extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with aq. 1 M $KHSO_4$ (3 × 50 mL), followed by brine (2 × 50 mL), dried (Na_2SO_4), filtrated, and evaporated under reduced pressure to give the bis-azide **7** as a yellowish oil in 98% yield (1.33 g, 4.9 mmol). R_f (CH_3Cl_3/MeOH/AcOH 89:10:1 v/v/v): 0.88; ^1H NMR (300 MHz, $CDCl_3$) δ = 3.69−3.59 (m, 8H, O−CH_2 (4 × 2H)), 3.55 (t (J = 6.0 Hz), 4H, ∼O−CH_2−CH_2−CH_2∼ (2 × 2H)), 3.40 (t (J = 6.5 Hz), 4H, CH_2-N_3 (2 × 2H)), 1.86 (quintet (J = 6.3 Hz), 4H, ∼O-CH_2−CH_2−CH_2∼ (2 × 2H)); ^{13}C NMR (75 MHz, $CDCl_3$) δ 70.3, 70.1, 67.7, 48.2, 29.0.

O-(3-Azidopropyl)-O′-(N-dansyl-aminopropyl)diethylene glycol ***8***. Hydrochloride **6** (1.0 g, 3.5 mmol) was dissolved in CH_2Cl_2 (30 mL) and to this solution Et_3N (1.1 mL, 8.0 mmol) followed by dansyl chloride (**11**) (1.0 g, 3.8 mmol) were added, and the obtained reaction mixture was allowed to stir for 3 h. Subsequently, the reaction mixture was concentrated *in vacuo*, and the residue was redissolved in EtOAc (50 mL), and this solution was washed with aq. sat. NH_4OAc (2 × 30 mL). Then, the EtOAc solution was dried (Na_2SO_4), filtrated, and evaporated *in vacuo* and the resulting residual oil was purified by column chromatography using a gradient of acetone (2.5% → 10% v/v) in CH_2Cl_2/Et_3N (100:0.1 v/v) as the eluents to afford azide **8** as a yellow oil in 60% yield (1.0 g, 2.1 mmol). R_f ($CHCl_3$/MeOH/AcOH 89:10:1 v/v/v): 0.75; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.53 (d (J = 8.5 Hz), 1H, dansyl-CH), 8.32 (d (J = 8.6 Hz), 1H, dansyl-CH), 8.24 (d (J = 7.3), 1H, dansyl-CH), 7.53 (m, 2H, dansyl-CH), 7.18 (d (J = 7.5), 1H, dansyl-CH), 5.66 (s, 1H, SO_2NH), 3.63 (m, 6H), 3.57−3.30 (m, 8H), 3.03 (m, 2H, CH_2-$NHSO_2$), 2.89 (s, 6H, $N(CH_3)_2$), 1.83 (quintet (J = 6.3), 2H, ∼CH_2−CH_2−CH_2−N_3), 1.66 (m, 2H, ∼CH_2−CH_2−CH_2−$NHSO_2$∼); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 151.9, 135.0, 130.1, 129.9, 129.7, 129.4, 128.2, 123.2, 119.1, 115.1, 70.6, 70.5, 70.3, 70.1, 70.0, 67.8, 48.5, 45.4, 42.2, 29.1, 28.6.

O-(3-Azidopropyl)-O′-(N-(5(6)-carboxyfluoresceinyl)-aminopropyl)diethylene glycol ***9***. Hydrochloride **6** (90 mg, 0.32 mmol) was dissolved in CH_3CN/DMF (10 mL, 9:1 v/v) and to this solution, DIPEA (220 μL, 1.26 mmol) followed by active ester 5(6)-carboxyfluorescein-OSu **12** (100 mg, 0.21 mmol) were added. The reaction mixture was stirred for 3 h and subsequently concentrated *in vacuo*. The residue was redissolved in EtOAc (10 mL) and the solution was washed with aq. 1 M $KHSO_4$ (2 × 8 mL). The EtOAc solution was dried (Na_2SO_4), filtrated, and evaporated under reduced pressure and the obtained residue was further purified by column chromatography (CH_2Cl_2/MeOH 92.5:7.5 v/v) to give compound **9** as a greenish oil in 76% yield (94 mg, 0.16 mmol). R_f ($CHCl_3$/MeOH/AcOH 89:10:1 v/v/v): 0.40; ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$ 95:5 v/v) δ = 8.35 (s), 8.10 (d (J = 8.0 Hz)), 7.98 (s, 1H, NH), 7.87 (m), 7.75 (m), 7.58 (s), 7.16 (d (J = 7.9 Hz)), 6.74−6.55 (m, 2H), 6.55−6.37 (m, 4H), 4.46 (broad s, 2H, ∼OH), 3.68−3.30 (m, 14H, O−CH_2 (6 × 2H)/NH−CH_2), 3.25 (m, 2H, N_3−CH_2), 1.86−1.70 (m, 4H, ∼CH_2−CH_2−CH_2∼); ^{13}C NMR (75.5 MHz, $CDCl_3/CD_3OD$ 95:5 v/v) δ = 169.4, 169.3, 166.4, 160.4, 152.9, 152.8, 140.6, 136.3, 134.1, 129.2, 127.7, 125.6, 125.0, 123.9, 123.3, 113.2, 109.9, 103.0, 70.3, 70.0, 69.9, 69.7, 67.9, 48.3, 38.8, 28.9, 28.8, 28.7, 28.4.

Nisin-dansyl ***13***. A stock solution of dansyl-azide **8** (3.8 mg, 7.9 μmol) in DMF (144 μL) and a stock solution of $CuSO_4 \cdot 5H_2O$ (13.2 mg, 52.9 μmol) in H_2O (2.64 mL) were prepared. Then, nisin-alkyne **2** (1.7 mg, 0.5 μmol) was dissolved in *tert*-BuOH/H_2O (450 μL, 1:1 v/v) and to this solution, an aliquot of the dansyl-azide stock solution (10 μL, 0.55 μmol, 1.10 equiv) and $CuSO_4$ stock solution (50 μL, 1.00 μmol, 2.0 equiv), respectively, were added, followed by the addition of solid sodium ascorbate (0.49 mg, 2.5 μmol, 5.0 equiv). This reaction mixture was heated to 80 °C by means of microwave irradiation during 20 min. Subsequently, the reaction mixture was diluted with buffer A (1.5 mL) and the product was purified using semipreparative HPLC. The pure fractions were identified by analytical HPLC and lyophilized to obtain nisin conjugate **13** as a white fluffy powder in 52% overall yield (1.0 mg). R_t 24.47 min; MALDI-TOF-MS calcd. for $C_{168}H_{266}N_{48}O_{41}S_8$: 3867.80, found m/z 3869.53 $[M+H]^+$, 3891.42 $[M+Na]^+$, 3906.25 $[M+K]^+$.

Nisin-CF ***14***. A stock solution of compound **9** (2.6 mg, 4.2 μmol) in DMF (76 μL) and a stock solution of $CuSO_4 \cdot 5H_2O$ (18.3 mg, 73.3 μmol) in H_2O (3.66 mL) were prepared. Then, nisin-alkyne **2** (1.7 mg, 0.50 μmol) was dissolved in *tert*-BuOH/H_2O (450 μL, 1:1 v/v) and to this solution, an aliquot of the stock solution containing compound **9** (10 μL, 0.55 μmol, 1.10 equiv), $CuSO_4$ stock solution (50 μL, 1.00 μmol, 2.0 equiv), respectively, were added, followed by the addition of solid sodium ascorbate (0.49 mg, 2.50 μmol, 5.0 equiv). The obtained reaction mixture was heated to 80 °C by microwave irradiation during 20 min. Then, the reaction mixture was diluted with buffer A (1.5 mL) and the product was purified using semipreparative HPLC. The pure fractions were identified by analytical HPLC and lyophilized to obtain nisin conjugate **14** as a yellowish fluffy powder in 31% overall yield (0.6 mg). R_t 24.93 min; MALDI-TOF-MS calcd. for $C_{177}H_{265}N_{47}O_{45}S_7$: 3992.79, found m/z 3993.5 $[M+H]^+$, 4014.82 $[M+Na]^+$, 4029.92 $[M+K]^+$.

Nisin-dimer ***15***. A stock solution of bis-azide **7** (3.2 mg, 11.8 μmol) in DMF (235 μL) and a stock solution of $CuSO_4 \cdot 5H_2O$ (16.4 mg, 65.7 μmol) in H_2O (1.64 mL) were prepared. Then, nisin-alkyne **2** (3.4 mg, 1.0 μmol, 1.0 equiv) was dissolved in *tert*-BuOH/H_2O (450 μL, 1:1 v/v) and to this solution an aliquot of the bis-azide stock solution (10 μL, 0.50 μmol, 1.0 equiv of azide) and $CuSO_4$ stock solution (50 μL, 2.0 μmol, 2.0 equiv), respectively, were added, followed by the addition of solid sodium ascorbate (0.99 mg, 5.0 μmol, 5.0 equiv). The obtained reaction mixture was heated to 80 °C by microwave irradiation during 20 min. Then, the reaction mixture was diluted with buffer A (1.5 mL) and the product was purified using semipreparative HPLC. The pure fractions were identified by analytical HPLC and lyophilized to obtain nisin dimer **15** as a white fluffy powder in 37% overall yield (1.3 mg). R_t 24.60 min; MALDI-TOF-MS calcd. for $C_{302}H_{486}N_{92}O_{75}S_{14}$: 7054.60 (average mass), found m/z 7055.5 $[M+H]^+$, 7077.3 $[M+Na]^+$, 7093.0 $[M+K]^+$.

Minimal Inhibitory Concentration Assays. Strains used for determination of antimicrobial activity included the American Type Culture Collection (ATCC) strain *Staphylococcus aureus* ATCC 259923 and *Bacillus subtilis*. The minimal inhibitory concentration (MIC) of each peptide was determined using a broth microdilution assay adapted from a literature procedure as previously described by Hancock.[34] Peptide stock solutions were prepared at a concentration of 100 μM peptide in 0.2% bovine serum albumin (BSA) and 0.01% acetic acid. Serial 3-fold dilutions of the peptides were made in 0.2% BSA and 0.01% acetic acid. Two wells were filled with 50 μL of the test bacterium in Mueller-Hinton broth (for *S. aureus*) or in tryptic soya broth (for *B. subtilis*) to a final concentration of 2 × 10^6 CFU/mL and 50 μL of the peptide solution with a different concentration was added, while the third well was filled with medium (without bacteria) as the negative control. The concentration range of the peptides was between 0 and 50 μM, with the following final concentrations: 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.07, and 0 μM. After incubation for 24 h at 37 °C at 120 rpm in a Certomat incubator, the OD at 630 nm was measured. The MIC (expressed in μM) of each peptide was read as the lowest concentration of peptide that was able to inhibit visible bacterial growth (see Supporting Information). All measurements were performed in duplicate.

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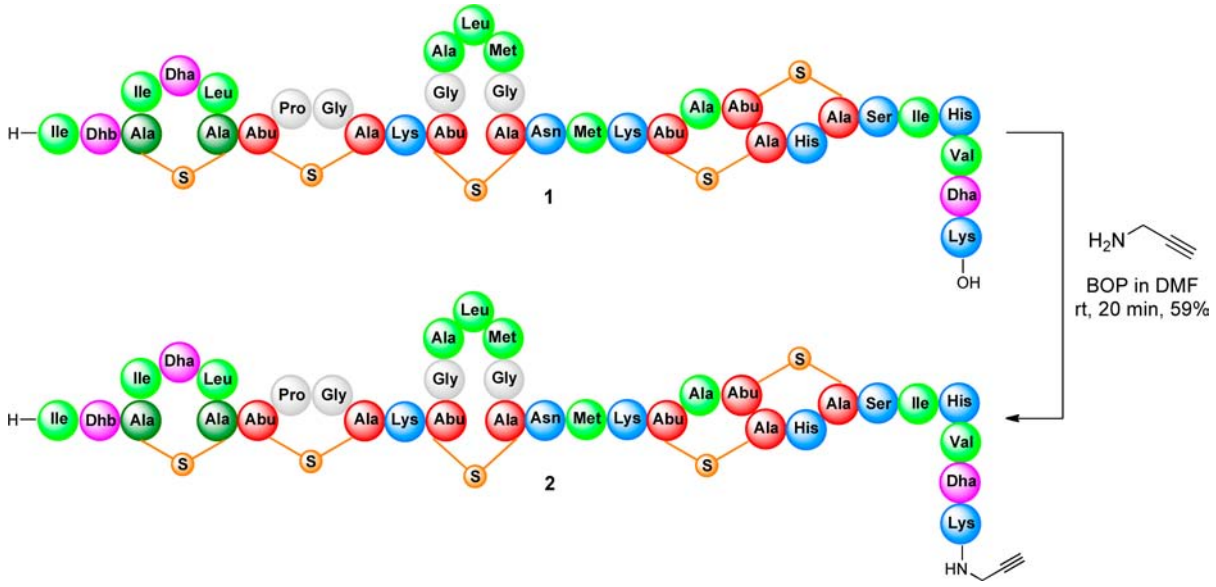
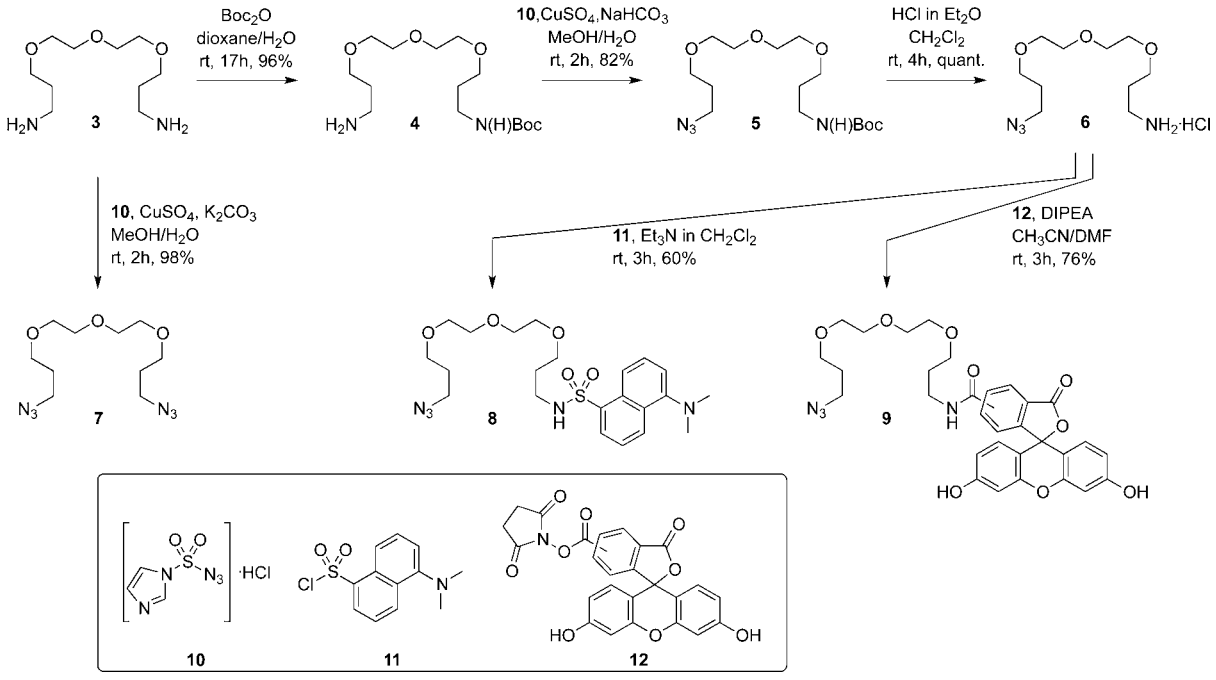
Scheme 1. Synthesis of Nisin-Alkyne 2



Scheme 2. Synthesis of Azides 7−9



Vesicle Leakage Experiments. Carboxyfluorescein (CF) loaded large unilamellar vesicles (LUVs) were prepared and used in a model membrane leakage experiment according to a literature procedure.[35] The peptide-induced leakage of CF from the vesicles was monitored by measuring the increase in fluorescence intensity at 515 nm (excitation at 492 nm) on a SPF 500 C spectrophotometer (SLM instruments Inc., USA). A solution (1.0 mL) of CF-loaded vesicles (20 μM final concentration) in buffer (50 mM TRIS-HCl, pH 7.0, containing 100 mM NaCl) was added to a quartz cuvette and fluorescence was measured (A_0). After 20 s, a sample solution containing the peptide of interest (1 μL from a freshly prepared stock solution of 1 μM resulted in a final concentration of 1 nM) was added and peptide-induced membrane leakage was followed during 60 s (A_{60}), after which a stock solution of Triton-X (10 μL of a stock solution (20%) resulted in a final concentration of 0.2%) was added to induce total leakage of the vesicles (A_{Total}). The % of peptide-induced leakage was calculated by: $((A_{60} - A_0)/(A_{Total} - A_0)) \times 100\%$. The reported value is the average value of two independent determinations.

RESULTS AND DISCUSSION

Chemistry. Nisin was purified from a commercial available nisin preparation, which contains only 2.5% nisin, via an extraction procedure as was recently reported.[31] Nisin **1** was used as such (this is without any protection groups) and its C-terminal carboxylate was reacted with a 25-fold excess of inexpensive propargylamine in the presence of BOP as the coupling reagent (Scheme 1). This reaction can be easily scaled

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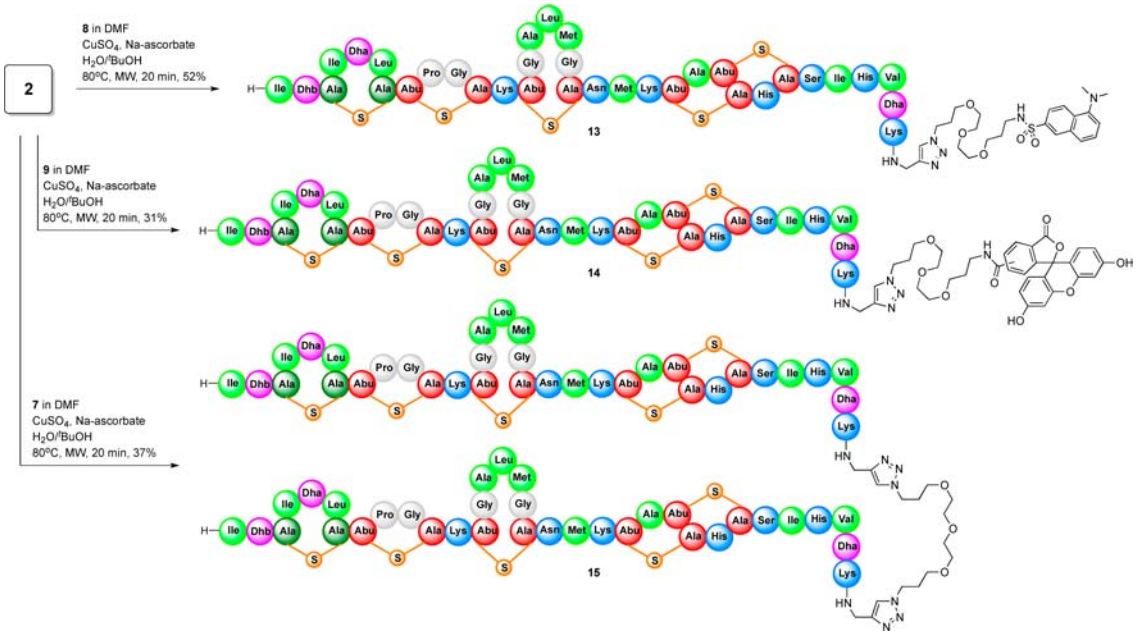
Scheme 3. Synthesis of Nisin-Dansyl 13, Nisin-CF 14, and Nisin Dimer 15, via Cu(I)-Catalyzed Click Chemistry



up and nisin alkyne **2** was conveniently isolated by preparative HPLC to give the corresponding peptide alkyne in good yield (59%) and high purity (>90%) (see Supporting Information). For labeling nisin-alkyne **2** with fluorescent reporter molecules, it was decided to synthesize the fluorescent azides **8** and **9** (Scheme 2), representing a dansyl- and a carboxyfluorescein-derivative, respectively. A diethylene glycol spacer was introduced to avoid any steric hindrance and might contribute to improving the solubility of the azide derivatives.

Their synthesis started with the monoprotection of *O*,*O*′-bis(3-aminopropyl)diethylene glycol **3** in the presence of Boc_2O to give carbamate **4** in excellent yield (96%). For the conversion into the corresponding azide, **4** was treated with imidazole-sulfonyl azide hydrochloride (**10**) as the diazotransfer reagent in the presence of Cu^{2+} according to Goddard-Borger and Stick,[33] and azide **5** was obtained in a high yield of 82%. After the quantitative removal of the Boc-group by treatment of **5** with a solution of dry HCl in diethyl ether, hydrochloride **6** was reacted with dansyl chloride **11** or the active *N*-hydroxysuccinimidyl ester **12** to give dansyl-azide **8** and carboxyfluorescein-azide **9**, in a yield of 60% and 76%, respectively, as shown in Scheme 2. In parallel with these syntheses, bis-azide **7** was prepared from bis-amine **3** in the presence of **10**/Cu^{2+} in a nearly quantitative yield (98%).

With the azides **7**–**9** in hand, the stage was set to couple them to nisin-alkyne **2** via CuAAC-based bioconjugation (Scheme 3). In a first attempt, dansyl-azide **8** was reacted with alkyne **2** by using 0.1 equiv $CuSO_4$ and 0.5 equiv sodium ascorbate in *tert*-BuOH/H_2O at 80 °C by microwave irradiation for 20 min.[36] Under these reaction conditions, incomplete conversion of the starting materials was observed by analytical HPLC (data not shown). Prolonging the reaction time to 60 min did not increase the conversion; instead, nisin degradation products were observed. Gratifyingly, increasing the number of equivalents of $CuSO_4$ and sodium ascorbate improved the conversion, and best results were obtained when 2 equiv $CuSO_4$ and 5 equiv sodium ascorbate were used, and heating the reaction mixture to 80 °C by microwave irradiation for 20 min. These optimized reaction conditions were applied, and nisin-alkyne **2** was conjugated to dansyl-azide **8** and carboxyfluorescein-azide **9** to give, after preparative HPLC, the fluorescent nisin conjugates **13** and **14**, respectively, in good to acceptable yields (52% and 31%) and in excellent purity. The same protocol was used for the synthesis of nisin dimer **15** in which bis-azide **7** was reacted with alkyne **2** and conjugate **15** was isolated in 37% yield as a single peak according to analytical HPLC (see Supporting Information).

Growth Inhibition Assays. As a first test to evaluate the biological activity of the newly synthesized nisin-analogs, a growth inhibition assay was performed. Two different Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, were used and the minimal inhibitory concentration (MIC) was determined for each construct using a broth microdilution assay,[34] and the obtained MIC-values are given in Table 1. As the positive control, nisin **1** was used. Generally, the obtained MIC-values of the nisin conjugates **2**, and **13**–**15** were in the same range as nisin. Interestingly, the activity of nisin-dansyl **13** was slightly improved (one dilution-step (1:3) lower) against *B. subtilis*, while the activity of nisin-CF **14** was approximately 10-fold less against *S. aureus* (Table 1). These results showed that C-terminal modifications were well-tolerated since the found MIC-values were in the same range as unmodified nisin. The

Table 1. Biological Activities of Nisin and the Synthesized Nisin Conjugates

	MIC (μM)[a]	
Peptide	*B. subtilis*	*S. aureus*
Nisin (**1**)	0.21–0.62	1.85–5.56
Nisin-alkyne (**2**)	0.21–0.62	1.85–5.56
Nisin-dansyl (**13**)	0.07–0.21	1.85–5.56
Nisin-CF (**14**)	0.62–1.85	16.7–50
Nisin-dimer (**15**)	0.62–1.85	1.85–5.56

[a]Antimicrobial activity is expressed as the Minimal Inhibitory Concentration (MIC). The concentration range of the nisin derivatives was 0–50 μM in a broth microdilution (serial 3-fold dilutions) assay.[34]

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importance of C-terminal fluorescently labeled nisin analogues[12] as molecular tools to shed more light on its mechanism of action has been recently shown in studies by Scherer et al.[24] and Desobry et al.[25]

Nisin's mode of action (vide supra) is based on the interaction with lipid II followed by pore formation. This pore-complex is believed to be a specific complex formed by eight nisin and four lipid II molecules in a 2:1 nisin:lipid II stoichiometry.[11] Based on this model, nisin dimer **15** was designed and it was expected that this dimer should be more active than the nisin monomer due to multivalency.[37] Apparently, formation of the nisin pore-complex did not benefit from the presence of nisin dimer **15**, since both monomer and dimer were equally active expressed as their MIC values. Nevertheless, retention of leakage activity was observed despite the increased steric hindrance caused by dimerization.

Membrane Leakage Assays. In a second biochemical assay, nisin conjugates **2** and **13**−**15** were tested for their interaction with model membrane systems.[35] For this purpose, large unilamellar vesicles (LUVs) were loaded with carboxyfluorescein (CF) as the fluorophore, and the ability of the nisin conjugates to induce membrane permeability was measured by monitoring the release of CF by fluorescence spectroscopy. LUVs composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), a zwitterionic lipid mimicking mammalian membranes, spiked with 0.2% lipid II as the natural target of nisin, were treated with nisin **1** (at 1 nM) and ∼46% of CF leakage was observed (as shown in Figure 2). Membrane lysis induced

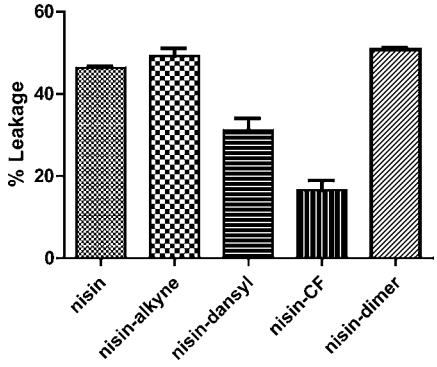

Figure 2. Lysis of large unilamellar vesicles expressed in percentage of leakage induced by nisin and nisin-conjugates (at 1 nM final peptide concentration). A lower leakage % indicates a less efficient membrane permeabilization.

by nisin-alkyne **2**, nisin-dansyl **13**, nisin-CF **14**, and nisin-dimer **15** (all derivatives were measured at 1 nM) was found to be ∼49%, ∼31%, ∼16%, and ∼51%, respectively. Interestingly, a slightly increased pore-formation activity was observed for nisin-dimer **15**, while the fluorescently labeled nisin conjugates **13** and **14** induced less CF-leakage, compared to nisin **1**. The concentration of conjugates **13** and **14** were too low (1 nM) to interfere with the fluorescence signal of carboxyfluorescein (50 mM).

As mentioned above, nisin binds lipid II embedded in the bacterial cell membrane via the N-terminal AB-ring fragment, and directs its C-terminal part into the phospholipid membrane to form pores and thereby lyse the bacterial cell. However, there are nisin mutants with an altered or even truncated C-terminus,[38−40] and other related lantibiotics like mutacin 1140, mutacin B-Ny266 and mersacidin,[13] with a similar AB-ring fragment as nisin, that do not have pore-forming abilities, but still are potent antibiotics. This alternative mechanism of antimicrobial activity has been unraveled by Breukink et al.[12] since lantibiotics with a functional AB-ring fragment bind lipid II and thereby interfere with cell division and act as a bactericidal agent to kill bacteria. Generally, the newly synthesized nisin conjugates **2** and **13**−**15** retained their biological activity compared to nisin **1**. However, subtle differences in activity may be explained by a combined interpretation of the measured MIC-values (Table 1) and the membrane permeabilization assay (Figure 2). Most pronounced in this case was the reduced activity of nisin-CF **14** since a 10-fold lower activity against *S. aureus* as well as the lowest pore-forming capacity might suggest that the C-terminal modification with a carboxyfluorescein moiety interfered with lipid II binding and membrane insertion.

CONCLUSIONS

In conclusion, a versatile synthesis route toward C-terminally functionalized nisin derivatives was developed. The introduction of an alkyne moiety enabled access to a wide variety of nisin conjugates, since a diverse set of functionalized azides can by efficiently coupled via the Cu(I)-catalyzed alkyne−azide cycloaddition reaction. The newly synthesized nisin conjugates were active in a bacterial growth inhibition assay as well as in a membrane permeabilizing assay. The retained biological activities suggest that these nisin conjugates can be used as—conveniently accessible—fluorescent probes as molecular tools to increase the insight of the mechanistic details to understand the mode of action of nisin and other related lantibiotic peptides. Moreover, the synthesis of well-defined covalent nisin dimers has been demonstrated for the first time and it might prove interesting to investigate different orientation and multivalency patterns of the nisin constructs to improve antimicrobial activity. Furthermore, to address the interest in surface modification with antimicrobial peptides,[41,42] the C-terminal conjugation of nisin-alkyne to azide-functionalized surfaces or coatings is highly recommended.

ASSOCIATED CONTENT

Supporting Information

Copies of the ^{1}H and ^{13}C NMR spectra of compounds **5**−**9**, copies of ESI-MS spectra of **8** and **9**, and analytical HPLC and MALDI-TOF-MS data of nisin-conjugates **2** and **13**−**15**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Jack C. Slootweg and Steffen van der Wal contributed equally to this work.

Notes

The authors declare no competing financial interest.

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