Regioselective modification of carbohydrates for their application as building blocks in synthesis
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Chapter 5

The synthesis and antibacterial activity of streptozotocin and its analogues

*Streptozotocin has been identified as an antibiotic. It also has antitumor activity, and has been approved by the FDA for treating metastatic cancer of the pancreatic islet cells. Although it is a broad spectrum antibiotic, it has specific toxicity to \( \beta \) cells of the pancreas in mammals, and therefore the application of Streptozotocin as an antibiotic is very limited. Here, we designed 3 streptozotocin analogues, in an attempt to retain the antibacterial activity and eliminate the cytotoxicity to \( \beta \) cells. Although the modifications were successfully carried out, tests show that when the toxicity drops, also the antibacterial activity drops.*

*The work in this chapter has been carried out in collaboration with L. Yakovlieva (Chemical Biology, Stratingh Institute for Chemistry). She carried out the bacterial activity tests and wrote paragraph 5.5.*
5.1 Introduction

Streptozotocin (STZ) (2-deoxy-2-(3-methyl-3-nitrosourea)-1-D-glucopyranose) is a naturally occurring carbohydrate, produced by Streptomyces achromogenes (Fig 1). Streptozotocin, already discovered in the 1960’s, exists as a 50-50 mixture of its α and β anomers. STZ is most stable at pH 4.5 and degrades rapidly in alkaline solutions. It was shown to exhibit broad spectrum antibacterial activity and possesses antitumor and mutagenic properties. Although initially developed as an antibiotic, and not particular toxic for mammalian cells in general, STZ was found to be very toxic to the insulin-producing β-cells of the pancreas. Therefore antibiotic development was terminated and STZ is currently applied to generate experimental animal models of diabetes. STZ is an N-acetyl glucosamine analogue that inhibits DNA synthesis in bacterial and mammalian cells. In bacteria, STZ accumulates intracellularly in the toxic phosphorylated form, and results in bacteriostasis. Diazomethane is generated by hydrolysis of STZ, leading to DNA damage. At low intracellular concentrations, septum formation in the bacteria is affected, the bacteria grow into filaments, and some surviving bacteria are also mutagenized. The mode of action of STZ to kill β cells is reasonably, though not fully, understood.

![Figure 1. Structure of streptozotocin](image)

5.2 The diabetogenic action of STZ

STZ is specifically toxic to pancreatic β cells that secrete insulin. This selectivity for pancreatic β cells has been associated with the 2-amino-2-deoxyglucoside, which appears to act as a carrier for the N3-methyl-N3-nitrosourea group. Due to the structural similarities with the chemical structure of glucose, STZ can also be transported into the β cell by the glucose transport protein 2 (GLUT2). In contrast to glucose, STZ is not recognized by other glucose transporters. Since GLUT2 is primarily found in cellular membranes of pancreatic β cells, this explains why STZ is selectively toxic to the pancreas. Because of this, STZ has been used extensively to create animal models...
of type1 diabetes.\textsuperscript{13} It is now also in use (and FDA approved) for islet-cell carcinomas and malignant carcinoid tumors in humans.

There are three postulated mechanisms by which STZ may work, when STZ enters into pancreatic β-cells. (1) As with other alkylating agents of the nitrosourea class, the DNA methylating activity of the methylimidazotetrazolo unit of STZ,\textsuperscript{14} leads to DNA fragmentation. This in turn induces activation of poly(ADP-ribose) polymerase (PARP), which tries to repair damaged DNA, and this results in overstimulation of DNA repair mechanisms leading to depletion of cellular NAD\textsuperscript{+} and ATP.\textsuperscript{3} Consequently the insulin-secreting cells undergo necrosis.\textsuperscript{15} Nicotinamide can inhibit the enzyme poly(ADP-ribose) polymerase (PARP). At the same time nicotinamide, as the precursor of NAD\textsuperscript{+}, can improve the levels of intracellular NAD\textsuperscript{+}. Therefore, administration of nicotinamide before induction of diabetes by STZ in rats, prevents depletion of NAD\textsuperscript{+} (and ATP), and protects the pancreatic β-cells from the toxicity of STZ.\textsuperscript{3} (2) STZ can also act as a nitric oxide donor in pancreatic cells.\textsuperscript{13} Biochemical evidence suggests that STZ does indeed increase NO levels in pancreatic β cells.\textsuperscript{13} The activity of guanylyl cyclase is increased and cGMP is formed, indicating the actions of NO. NO inhibits aconitase activity, leading to DNA alkylation and damage.\textsuperscript{3} (3) Another mechanism has been proposed recently and is called the “O-GlcNAc-dependent model” of STZ toxicity.\textsuperscript{13} Protein O-GlcNAcylation plays an important role in modification of serine/threonine residues in higher eukaryotes. O-GlcNAcylation has shown to be related to diverse cellular processes including insulin secretion. The enzyme O-GlcNAcase is responsible for the hydrolysis of O-GlcNAcylated proteins to the free protein and GlcNAc. On the contrary, O-GlcNAc transferase is in charge of transferring GlcNAc to serine/threonine on proteins. Millimolar concentrations of STZ increases protein O-GlcNAcylation and abundant O-GlcNAc transferase was observed in the pancreas, this causes protein hyper-O-GlcNAcylation, indicating O-GlcNAcase was inhibited. All these data showed that STZ inhibits the activity of the glycoside hydrolase O-GlcNAcase, leading to hyper-O-GlcNAcylation. This triggers activation of stress pathways results in β cell apoptosis.\textsuperscript{13}

5.3 Modification of streptozotocin at C3

In 1972, B. Bannister of the company Upjohn reported some modifications of streptozotocin and their antibiotic activities. Fully acetylated streptozotocin has no antibacterial activity, but shows an even increased inhibition of the growth of L-1210 cells (L1210 is
a mouse lymphocytic leukemia cell line which is derived from the ascitic fluid of 8-month-old female mice) compared to STZ. Furthermore, also replacement of the methyl group at N\(^3\) of the nitrosourea by an ethyl or n-butyl substituent results in the loss of antibacterial activity. Inversion of the 4-hydroxyl group of streptozotocin (so going from gluco to galacto configuration) leads to the loss of antibacterial activity at least against *Proteus vulgaris*. Inversion at C\(_2\) (so going from gluco to manno configuration) reduces the antibacterial activity markedly as well. Kimmura et al. reported that O-alkylation of the anomic hydroxyl of STZ improves the antitumor activities and reduces toxicity, but leads to a loss of the antibacterial and diabetogenic activity. Bannister’s research also revealed that methylation of the anomic hydroxyl group of STZ eliminates antibacterial activity, and \(\alpha\)-methyl STZ is twice as active as the \(\beta\)-methyl STZ against cultures of leukemia L1210. The cytotoxicity of \(\beta\)-methyl STZ was identical to STZ.

The aim of the project described in this thesis is that we attempted to retain the antibacterial activity of streptozotocin but eliminate the cytotoxicity to \(\beta\) cells. We tried to achieve this goal by aiming for uptake of STZ by bacteria, that in general are quite promiscuous in the uptake of carbon sources, but not via mammalian GLUT2. Slight modifications of the carbohydrate part of STZ might allow this distinction. Up till now, some STZ analogues have been reported. Although the galacto- and manno-pyranoside derivatives of STZ have been prepared and studied (with little success, see above), the allopyranoside analogue has not. This is for obvious reasons; allosamine is a rare sugar and only available by multistep synthesis (see Chapter 3 though). As GLUT2 is not expected to accept allose-configured carbohydrates as substrates, but bacteria are known to contain allose transporters, this seemed a viable strategy. Therefore we designed allo-streptozotocin, its synthetic precursor keto-streptozotocin, and 3-deoxy streptozotocin as target compounds.

![Figure 2. Structure of the designed molecules](image)

5.4 Preparation of streptozotocin and its analogues
Currently, there are two different synthetic routes to streptozotocin, apart from production by fermentation that has shown to be inconvenient. In a first approach, glucosamine hydrochloride is treated with methyl isocyanate under basic conditions to give N-carbamyl-N’-methyl-D-glucosaminide (“the 3N-methyl urea derivative of glucosamine”). Nitrosation of 1 is subsequently carried out with sodium nitrite in acidic medium to give streptozotocin (Scheme 2a). This route has major drawbacks as the nitroso group can not only be introduced at the required N²-position, but also at the nitrogen of glucosamine (N¹, shown in scheme 2a). In addition, working with methyl isocyanate is not attractive. An alternative synthetic route has been proposed for the regioselective synthesis of streptozotocin. This route involves the use of N-nitrosocarbamates as intermediates. Coupling with glucosamine in the presence of an organic base gives streptozotocin (Scheme 2b). Initially, we studied the first method, though avoiding the use of methyl isocyanate. As mentioned, nitrosation of 1 generated two isomers, with Rf values too close to separate them efficiently. Therefore, we abandoned this way and focused our attention on the reaction of glucosamine with N-nitrosocarbamates (Scheme 2).

Scheme 2. The synthesis of Streptozotocin according to literature
4-Nitrophenyl N-nitroso-N-methylcarbamate 6 was prepared by dissolving 4-nitrophenyl chloroformate in THF at 0 °C, followed by the addition of a methylamine solution in THF to afford 4-nitrophenyl methylcarbamate 5. To get 6, nitrosation of 5 with NaNO₂ was carried out in a mixture of DCM and 12 M HCl in water. Allosamine and lividosamine were synthesized according to the procedure described in the previous chapter. Glucosamine hydrochloride, allosamine hydrochloride and lividosamine hydrochloride were treated with 4-nitrophenyl N-nitroso-N-methylcarbamate 6 in the presence of i-Pr₂NEt in DMF to get the corresponding streptozotocin, allo-streptozotocin and deoxy-streptozotocin. The NMR and IR data of streptozotocin prepared in this way matched those obtained from commercial streptozotocin. Allo-streptozotocin and deoxy-streptozotocin were isolated as a mixture of the pyranose and furanose forms, with the pyranose form being the major. 3-keto glucosamine cannot be prepared from glucosamine directly, since the free amino group inhibits the catalyst. To obtain keto-streptozotocin 9, streptozotocin was therefore regioselectively oxidized with benzoquinone in the presence of Pd-catalyst, see chapter 3.. We were pleased to see that our palladium-catalyzed oxidation worked in the presence of a N-nitroso urea unit.
Scheme 3. The synthesis of STZ analogues
5.5 Antibacterial activity tests and discussion

Growth-based bacterial viability assay with streptozotocin derivatives

Introduction

In order to assess the impact of derivatization on bactericidal activity, streptozotocin derivatives were tested in a viability assay on E. coli cells. The assay was performed in rich medium, lysogeny broth (LB) as well as in minimal medium with N-acetylglucosamine or ribose as additives to study the effect of these compounds on the uptake of the streptozotocin-analogues. 23, 24

Growth curves rich medium: (L1 and L2 are duplicated)
E. coli viability with allo-streptozotocin in rich medium

E. coli viability with keto-streptozotocin in rich medium
Growth curves in minimal medium with GlcNAc (NAG)-presensitizing
E. coli viability with keto-streptozotocin in minimal medium with NAG-presensitizing

E. coli viability with deoxy-streptozotocin in minimal medium with NAG-presensitizing
Growth curves in minimal medium with ribose-presensitizing

Data interpretation:

The derivatization of the antibiotic streptozotocin at the C3 position was performed in order to reduce the toxicity for beta-cells while simultaneously retaining the antibacterial activity. This resulted in several compounds being made, namely allo-, keto- and deoxy-streptozotocin, with respect to modification introduced at C3 position. These three derivatives were tested in a viability assay alongside the original compound to compare their antibacterial properties. Regardless of the medium used, streptozotocin showed the highest bactericidal activity among the compounds which indicates that C3 modification interferes
either with the uptake of the compound by bacterial cells or with its mechanism of action. Among the derivatives of streptozotocin and under rich-medium conditions, keto-streptozotocin was the most active, whereas allo-streptozotocin showed minimal antibacterial effect and deoxy-streptozotocin showed no effect at all even at highest concentration tested (200 mg/L). In order to investigate whether the uptake mechanism of the compounds was similar to that of streptozotocin (through the $N$-acetylglucosamine uptake pathway), $N$-acetylglucosamine presensitized $E. coli$ cells were used in the same viability assay. Likewise the assay results in rich medium, streptozotocin showed the highest activity. Keto-streptozotocin also showed higher activity in the same concentration range compared to its effect on non-presensitized cells. In contrast, the antibacterial activity of allo- and deoxy-streptozotocin remained minimal even with the primed $N$-acetylglucosamine pathway. Hence, the conclusion could be drawn that while keto-streptozotocin seems to be taken up through the same sugar transport system as streptozotocin, allo- and deoxy- derivatives are not. Therefore, a different presensitizer was tested for its ability to induce the uptake of allo- and deoxy-streptozotocin, namely ribose. However, this did not lead to visible improvement of bactericidal activity suggesting that a different pathway must be employed to transport these compounds into the cells.

A different approach of assessing the antibacterial activity of the compounds is to perform MIC assays in which bacterial cells are incubated with potential inhibitors for much longer periods of time (2h in viability assay). That could allow more compound to penetrate the bacterial cells and reflect more accurately the antibacterial activity.

Test of our compounds on $\beta$ cells is still ongoing. The initial test with $\beta$ cells show that the the toxicity to $\beta$ cells for deoxy-streptozotocin and allo-streptozotocin is reduced, but keto-streptozotocin is still toxic to $\beta$ cells.

### 5.7 Conclusion

Streptozotocin has the best antibacterial activity, antibacterial activity of keto-streptozotocin is weaker than streptozotocin, deoxy-streptozotocin and Allo-streptozotocin are lower than keto-streptozotocin. The initial test on $\beta$ cells reveals the toxicity of deoxy-streptozotocin and allo-streptozotocin are reduced obviously, keto-streptozotocin still has the toxicity to $\beta$ cells, the overall conclusion is that modifications of streptosotocin are successful, but if the antibacterial activity drops, also the toxicity drops.
5.8 Experimental section

All solvents used for reaction, extraction, filtration, and chromatography were of commercial grade and used without further purification. Flash chromatography was performed on a Reveleis® X2 Flash Chromatography, using Grace® Reveleris Silica flash cartridges (4 grams, 12 grams, 15 grams, 24 grams, 40 grams, 80 grams and 120 grams) and Scorpius Diol (OH) 48 grams. ¹H-, ¹³C-, APT-, HSQC-, and COSY-NMR were recorded on a Varian AMX400 spectrometer (400, 100 MHz, respectively) using DMSO-δ6, D₂O or methanol-δ4 as solvent. Chemical shift values are reported in ppm with the solvent resonance as the internal standard (DMSO-δ6: δ 2.50 for ¹H, δ 39.52 for ¹³C, CD₃OD: δ 3.31 for ¹H, δ 49.15 for ¹³C; D₂O: δ 4.80 for ¹H). Data are reported as follows: chemical shifts (δ), multiplicity (s = singlet, d = doublet, dd = double doublet, ddd = double double doublet, t = triplet, app = apparent triplet, q = quartet, m = multiplet), coupling constants J (Hz), and integration. High Resolution Mass measurements were performed using a ThermoScientific LTQ OrbitrapXL spectrometer. Streptozotocin was obtained from Sigma-Aldrich (and prepared as well).

N-methyl 4-nitrophenyl carbamate (5)

Methyl amine (12 mmol, 6 ml of a 2 M solution in THF) was added to a solution of 4-nitrophenyl chloroformate (2.0 g, 9.9 mmol) in THF (100 ml) at 0°C. The reaction mixture was allowed to warm to r.t. and stirred overnight. The mixture was then concentrated and purified by flash chromatography on a silica cartridge with PE/EtOAc to obtain the product (1.07 g, 55%) as a white solid, m.p.: 144-146 °C (lit.²⁵ 145–146 °C); ¹H NMR (400 MHz, Chloroform-d) δ 8.24 (d, J = 9.0 Hz, 2H), 7.31 (d, J = 9.0 Hz, 2H), 5.07 (s, 1H), 2.93 (d, J = 4.7 Hz, 3H); ¹³C NMR (101 MHz, Chloroform-d) δ 156.0, 153.7, 144.7, 125.1, 121.9, 27.8. The NMR data match with the literature.²⁶

4-nitrophenyl N-nitroso-N-methylcarbamate (6)

To a solution of N-methyl 4-nitrophenyl carbamate (1 g, 5.1 mmol) in 20 mL DCM in a 100 mL flask, was added a solution of NaNO₂ (2.5 g, 36.2 mmol) in 20 mL of water. The mixture was chilled to 0 °C, and 5.2 mL conc. HCl aq was added dropwise and slowly (one drop per 3 seconds), upon which the color of the solution changed from yellow to green. The reaction was monitored by TLC, and after 3 h, a very small amount of starting material remained. Nevertheless, the DCM layer was separated, washed with water, dried with MgSO₄, and concentrated. Purification by flash chromatography on a silica cartridge with
pentane/DCM yielded a yellow solid (852 mg, 74\%); HRMS (ESI) m/z calcd for C_{8}H_{8}N_{3}O_{5} ([M+H]^+): 226.046 and C_{8}H_{7}N_{2}O_{4} ([M−NO]^+): 195.040; found: 225.979 and 195.039; 1^H NMR (400 MHz, Chloroform-d) δ 8.36 (d, J = 9.2 Hz, 2H), 7.53 (d, J = 9.1 Hz, 2H), 3.26 (s, 3H); 13C NMR (101 MHz, Chloroform-d) δ 154.8, 151.9, 145.9, 125.5, 122.3, 28.2. The NMR data match with the literature.^{27} IR (N−N=O): 1460 cm\(^{-1}\)

**Streptozotocin (3)**

Glucosamine hydrochloride (289 mg, 1.34 mmol) and 4-nitrophenyl \(N\)-nitroso-\(N\)-methylcarbamate (332 mg, 1.47 mmol) were dissolved in DMF (9.5 ml) at r.t., then the reaction mixture was cooled to 0 °C under N\(_{2}\) atmosphere, followed by the addition of diisopropylethylamine (279 μL, 1.6 mmol). The reaction mixture was stirred at 0 °C for 2 h, the DMF was evaporated and the residue purified by flash chromatography on a 12 g silica cartridge with DCM/MeOH, increasing the ratio of MeOH from 0% to 15% in 22 min, the product eluted at 7% MeOH to afford a white solid (259 mg, 73%). \([\alpha]_D^0 = +92^\circ\ (c = 0.0096, CH\(_3\)OH); HRMS (ESI) m/z calcd for C_{8}H_{16}N_{3}O_{7} ([M+H]^+): 266.098 and C_{8}H_{15}N_{2}O_{6} ([M−NO]^+): 235.092; found: 266.098 and 235.092; 1^H NMR (400 MHz, Methanol-d\(_4\)) δ 5.25 (d, J = 3.5 Hz, 1H), 3.99 (dd, J = 10.5, 3.5 Hz, 1H), 3.87 – 3.78 (m, 3H), 3.78 – 3.71 (m, 1H), 3.44 (t, J = 9.4 Hz, 1H), 3.16 (s, 3H). 13C NMR (101 MHz, Methanol-d\(_4\)) δ 155.4, 92.8, 73.4, 73.3, 72.3, 62.9, 57.3, 27.0. IR (N−N=O): 1450 cm\(^{-1}\). The NMR spectra of prepared and commercial 3 were compared and shown to be virtually identical.

**Allo-streptozotocin (7)**

Allosamine hydrochloride (393 mg, 1.82 mmol) and 4-nitrophenyl \(N\)-nitroso-\(N\)-methylcarbamate (451 mg, 2 mmol) were dissolved in DMF (12 ml) at r.t., then the reaction mixture was cooled to 0 °C under N\(_{2}\) atmosphere, followed by the addition of diisopropylethylamine (283 mg, 2.19 mmol). The reaction mixture was stirred at 0 °C for 2 h, the DMF was evaporated and the residue purified by flash chromatography on a 12 g silica cartridge with EtOAc/MeOH, increasing the ratio of MeOH from 0% to 10% in 22 min, the product eluted at 3% MeOH to afford a yellow solid (200 mg, 41%). NMR showed the major form is ρ-pyranose. \([\alpha]_D^0 = +10.7^\circ\ (c = 0.0101, CH\(_3\)OH); HRMS (ESI) m/z calcd for C_{8}H_{16}N_{3}O_{7} ([M+H]^+): 266.098 and C_{8}H_{15}N_{3}O_{6} ([M−H\(_2\)O]^+): 248.088; found: 266.097 and 248.087; 1^H NMR (400 MHz, Methanol-d\(_4\)) δ 4.96 (d, J = 8.3 Hz, 1H),
4.16 – 4.13 (m, 1H), 3.91 (dd, \(J = 8.3, 2.9\) Hz, 1H), 3.86 (dd, \(J = 11.7, 2.5\) Hz, 1H), 3.81 – 3.75 (m, 1H), 3.69 (dd, \(J = 11.6, 5.7\) Hz, 1H), 3.60 (dd, \(J = 9.6, 3.1\) Hz, 1H), 3.16 (s, 3H). \(^{13}C\) NMR (101 MHz, Methanol-\(d_4\)) \(\delta 153.5, 93.2, 74.4, 70.1, 67.4, 61.8, 55.6, 25.4\). IR (N-N=O): 1436 cm\(^{-1}\)

Deoxy-streptozotocin (8)

Lividosamine hydrochloride (154 mg, 0.771 mmol) and 4-nitrophenyl \(N\)-nitroso-\(N\)-methylcarbamate (191 mg, 0.848 mmol) were dissolved in DMF (5 ml) at r.t., then the reaction mixture was cooled to 0 °C under \(N_2\) atmosphere, followed by the addition of diisopropylethylamine (161 \(\mu\)L, 0.925 mmol), The reaction mixture was stirred at 0 °C for 2 h, the DMF was evaporated and the residue purified by flash chromatography on a 15 g silica cartridge with 100% EtOAc to afford the product (135 mg, 70%) as colorless oil. NMR showed the major form is \(\alpha\)-pyranose. \([\alpha]_D^0 = +34.7^\circ\) (c = 0.0117, CH\(_3\)OH); HRMS (ESI) m/z calcd for CsH\(_{15}\)N\(_3\)O\(_6\) ([M+H]+): 250.103 and CsH\(_{14}\)N\(_3\)O\(_5\) ([M-H\(_2\)O]+): 232.093; found: 250.103 and 232.093; \(^1\)H NMR (400 MHz, Methanol-\(d_4\)) \(\delta 5.17\) (d, \(J = 3.4\) Hz, 1H), 4.12 (dt, \(J = 12.7, 4.1\) Hz, 1H), 3.83 – 3.78 (m, 1H), 3.73 – 3.70 (m, 1H), 3.69 (q, \(J = 6.0\) Hz, 1H), 3.63-3.59 (m, 1H), 3.14 (s, 3H), 2.15 (dt, \(J = 11.6, 4.7\) Hz, 1H), 1.90 (q, \(J = 11.8\) Hz, 1H). \(^{13}C\) NMR (101 MHz, Methanol-\(d_4\)) \(\delta 154.7, 91.2, 74.1, 66.3, 62.9, 51.0, 34.1, 26.9\). IR (N-N=O): 1478 cm\(^{-1}\)

Keto-streptozotocin (9)

Streptozotocin (162 mg, 0.611 mmol) and benzoquinone (99 mg, 0.92 mmol) were dissolved in DMSO (2 mL). The catalyst [(neocuproine)PdOAc]_2OTf \(_2\) (15.7 mg, 2.5 mol%) was added and the mixture was stirred at r.t. for 1 h. Upon completion of the reaction (according to TLC), water (20 mL) was added and the mixture was lyophilized to afford the crude product. Subsequent purification by flash chromatography on a 12 g silica cartridge with pentane/EtOAc, increasing the ratio of EtOAc from 0% to 100% in 22 min, the product eluted at 100% EtOAc to afford a white solid (88 mg, 58%). HRMS (ESI) m/z calcd for CsH\(_{14}\)N\(_3\)O\(_7\) ([M+H]+): 246.083 and CsH\(_{14}\)N\(_3\)O\(_5\) ([M-H\(_2\)O]+): 246.073; found: 250.103 and 246.072. \(^1\)H NMR (400 MHz, Methanol-\(d_4\)) \(\delta 5.71\) (d, \(J = 4.1\) Hz, 1H), 4.94 (d, \(J = 4.1\) Hz, 1H), 4.40 (d, \(J = 9.7\) Hz, 1H), 4.00 (dt, \(J = 9.7, 3.3\) Hz, 1H), 3.90 – 3.85 (m, 2H), 3.16 (s, 3H). \(^{13}C\) NMR (101 MHz, Methanol-\(d_4\)) \(\delta 204.5, 95.3, 76.8, 73.9, 62.6, 61.9, 26.9\). IR (N-N=O): 1435 cm\(^{-1}\)
**Materials and Reagents.** *E. coli* TOP10 was obtained from the group of Biotransformations and Biocatalysis (GBB Institute, University of Groningen). Media, salts and additives were purchased from Sigma Aldrich, unless otherwise specified.

**Growth-based viability assay.** In order to determine the inhibitory effect of streptozotocin and its derivatives, the growth-based viability assay developed by C.L. Haynes et al.\(^2\) was used. The underlying principle of the assay is to estimate the viability of the cells after exposure to an inhibitory compound by the delay in the subsequent outgrowth in fresh medium. This effect is from the fact that the fewer the remaining cells, the longer it takes to reach a certain density threshold, indicating the bactericidal activity of the compound used. In the assay, bacterial cells were first exposed to the streptozotocin derivatives (either in rich or minimal medium) at room temperature and constant shaking to ensure good mixing. Afterwards, a small fraction of the exposure mixture was transferred into the fresh medium (yielding a 40x dilution and therefore alleviating the effect of the antibiotic) and the cells were incubated in a Biotek plate reader for 16 hours at 37°C with optical density measurements at 600 nm taking place every 20 min preceded by 30 s of shaking.

**Plate layout.** As recommended in the paper of Haynes et al.\(^2\) the original layout with water evaporation control was used in this work. For all experiments the following plate sections were included: calibration series with outgrowth in duplicates, 10x dilution series of the compound, exposure wells and after-exposure outgrowth wells in duplicates.
Viability assay in rich medium. Bacterial culture of *E. coli* TOP10 was grown in LB medium (37°C, shaking) until cells entered the exponential growth phase (OD$_{600}$ 0.3-0.5) and was diluted to working density of 0.05 (calibration curve series) and 0.1 (exposure). For exposure, 20 microliters of the 10x compound preparation in an appropriate solvent was transferred to 180 microliters of the bacterial culture, yielding 1x concentration of the compound for exposure. The plate was then incubated in the plate reader at room temperature and constant shaking for 2 hours. Afterwards, 5 microliters of the exposure mixture were added to 195 microliters of fresh LB to dilute the antibiotic and allow remaining bacterial cells to outgrow. The same outgrowth procedure was performed for the calibration series. As a last step, the plate was incubated for 16 hours in the plate reader at 37°C with OD$_{600}$ measurements every 20 min preceded by 30 s of shaking.

Viability assay in minimal medium on N-acetylglucosamine/ribose presensitized *E. coli* cells. Bacterial culture of *E. coli* TOP10 was pre-grown overnight in minimal medium containing 0.4% N-acetylglucosamine/ribose at 37 °C with shaking. Next day, cells were harvested and washed three times with Dulbecco’s phosphate saline buffer (DPBS) and resuspended in the same buffer to the original volume of the overnight culture. The resulting bacterial suspension was then used to inoculate minimal medium containing 1% glycerol which was further incubated at 37 °C and shaking. The bacterial culture was allowed to reach exponential phase densities of 0.3-0.5 and then was diluted to working densities of 0.05 (calibration curve) and 0.1 (exposure). Exposure and outgrowth procedures were performed in the same way as described in the section “Viability assay in rich medium”.

### 5.8 References


