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Comparative Life Cycle Assessment of Chemical and Biocatalytic 2′3′-Cyclic GMP-AMP Synthesis

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Life cycle assessments (LCAs) can provide insights into the environmental impact of production processes. In this study, a comparative LCA was performed for the synthesis of 2′3′-cyclic GMP-AMP (2′3′-cGAMP) in an early development stage. The cyclic dinucleotide (CDN) is of interest for pharmaceutical applications such as cancer immunotherapy. CDNs can be synthesized either by enzymes or chemical catalysis. It is not known which of the routes is more sustainable as both routes have their advantages and disadvantages, such as a poor yield for the chemical synthesis and low titers for the biocatalytic synthesis. The synthesis routes were compared for the production of 200 g 2′3′-cGAMP based on laboratory data to assess the environmental impacts. The biocatalytic synthesis turned out to be superior to the chemical synthesis in all considered categories by at least one magnitude, for example, a global warming potential of 3055.6 kg CO_2 equiv. for the enzymatic route and 56454.0 kg CO_2 equiv. for the chemical synthesis, which is 18 times higher. This study demonstrates the value of assessment at an early development stage, when the choice between different routes is still possible.

Introduction

Biocatalysis has developed into a mature and widely used technology for the synthesis of fine and bulk chemicals.[1] Especially for the synthesis of chiral compounds for pharmaceuticals, biocatalysis offers great advantages compared to chemical catalysis such as the high substrate specificity, mild reaction conditions, the use of water instead of solvents as reaction medium, and the regio- and stereoselectivity of enzymes.[2,3] For this reason, bioprocesses are often described as more sustainable and environmentally friendly. However, the production of enzymes and complex downstream processes can have a major impact on the overall process environmental footprint.[4,5] Therefore, a precise assessment of the environmental impact of manufacturing processes remains important.

Different methods for assessing the environmental impact of (bio)chemical processes have been established. The simplest approach is to calculate mass-based metrics, such as the E-factor developed by Roger Sheldon.[6] The E-factor is defined by the ratio of waste generated during production for a given quantity of product and is ideally 0. The extended E^+-factor additionally includes the energy consumed during production.[7] The pharmaceutical industry prefers to use the Process Mass Intensity (PMI) to evaluate the process greenness.[8] This is the ratio of all chemicals used to the mass of the isolated product. The advantage of these mass-based metrics is that they are easy to calculate and provide a quick initial environmental assessment of a process. However, a limitation is that they do not take into account the hazards and toxicities of the waste streams. In addition, all reactants are treated equally, regardless of the complexity of their production.

A significantly more detailed assessment of the environmental impact of a production process is possible using a life cycle assessment (LCA). The entire life cycle of a product is considered, from the extraction of raw materials, the manufacturing of the product to its use and disposal or recycling.[9] Compared to the mass-based metrics, a significantly larger amount of data is required, which can include all material and energy flows as well as the necessary equipment. The preparation is therefore much more labor-intensive and requires special expertise, which is why LCAs are rarely applied for early-stage processes, but rather for existing processes in industry. Nevertheless, early-stage process development studies have also been used to evaluate synthesis route alternatives and to identify ecologically inefficient process steps. Until now, only a few studies were published with a direct quantitative comparison of chemical and enzymatic processes for the synthesis of the same compound. This was demonstrated, for example, for the production of 7-aminocephalosporic acid (7-ACA) and by the comparative prospective LCA on the biocatalytic and chemical production of 1 g of the lactone β,δ-trimethyl-ε-caprolactones (TMCL).[10,11] Data from laboratory experiments on a small scale served as a calculation reference.[11] Interestingly, the results showed no difference between the synthesis routes on the climate change impact.

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Through a subsequent sensitivity analysis, parameters such as recycling solvents and enzymes as well as the use of electricity based on renewable energy sources were identified as beneficial to the overall assessment with reductions of up to 71% related to climate change. In another study, the economic and ecological sustainability impact of 11 substrates for the yeast-based fermentative citric acid production was evaluated.[12] Next to the substrates, different process modes were investigated. The evaluation showed that repeated batches with raw glycerol provided the best environmental performance. These studies show that LCAs can serve as a useful decision-making tool at an early stage of process development.

In this study, LCA is used as comparative assessment method to evaluate synthesis routes of the cyclic dinucleotide (CDN) 2′3′-cyclic GMP-AMP (2′3′-cGAMP). CDNs are currently of great interest in immune-oncology research and development due to its ability to induce type-I interferon release after binding to the stimulator of interferon genes (STING) receptor at the endoplasmic reticulum.[13,14] In mammalian cells, 2′3′-cGAMP is produced in the presence of cytosolic DNA from adenosine 5′-triphosphate (ATP) and guanosine 5′-triphosphate (GTP) by the cyclic GMP-AMP synthase (cGAS). It serves as a second messenger and is part of the innate immune system.[15,16] In recent years, various CDN derivatives have been synthesized with improved properties and thus potential application as pharmaceutical compounds. Different routes exist for their synthesis, based on either chemically or enzymatically catalyzed reactions.[17] The chemical synthesis of 2′3′-cGAMP shown in Figure 1, for example, consists of more than eight steps starting from phosphoramidites and yields 5% at a mg scale.[18,19] The biocatalytic one-step synthesis of 2′3′-cGAMP shown in Figure 2 from nucleoside triphosphates using cGAS was also investigated on a mg scale with a yield of 95% from 0.5 mM substrate.[20]

The synthesis routes differ significantly from each other and therefore lead to different process characteristics. The chemical synthesis results in a significantly lower yield, but the reaction mixture is already highly concentrated in the last step and requires only a few purification steps.[21] In contrast, biocatalysis results in a high reaction yield, but in a low titer (310 mg L^{-1}), and thus a more complex downstream processing is required to isolate the polar hydrophilic product from the aqueous reaction mixture.[17] It is therefore not possible to predict from the process parameter which of the processes is more sustainable at the current development stage and which key parameters have a significant influence.

For this reason, we have prepared a comparative LCA for the biocatalytic and chemical synthesis of 2′3′-cGAMP and determined the impacts on global warming potential (GWP) human health, ecosystems, resource scarcity, and water use. The production of 200 g 2′3′-cGAMP in both routes is compared, which is within the range required for preclinical or phase I clinical studies.[22] In the case of biocatalytic synthesis, the calculation was based on own laboratory data of mg scale, which were scaled up. Individual LCAs were created for the substrates ATP, GTP, and yeast extract based on patents. For the chemical synthesis of 2′3′-cGAMP, a synthesis protocol by Gaffney and Jones on a gram scale served as the basis for the calculation,[21] which was similarly scaled up. For this LCA study,

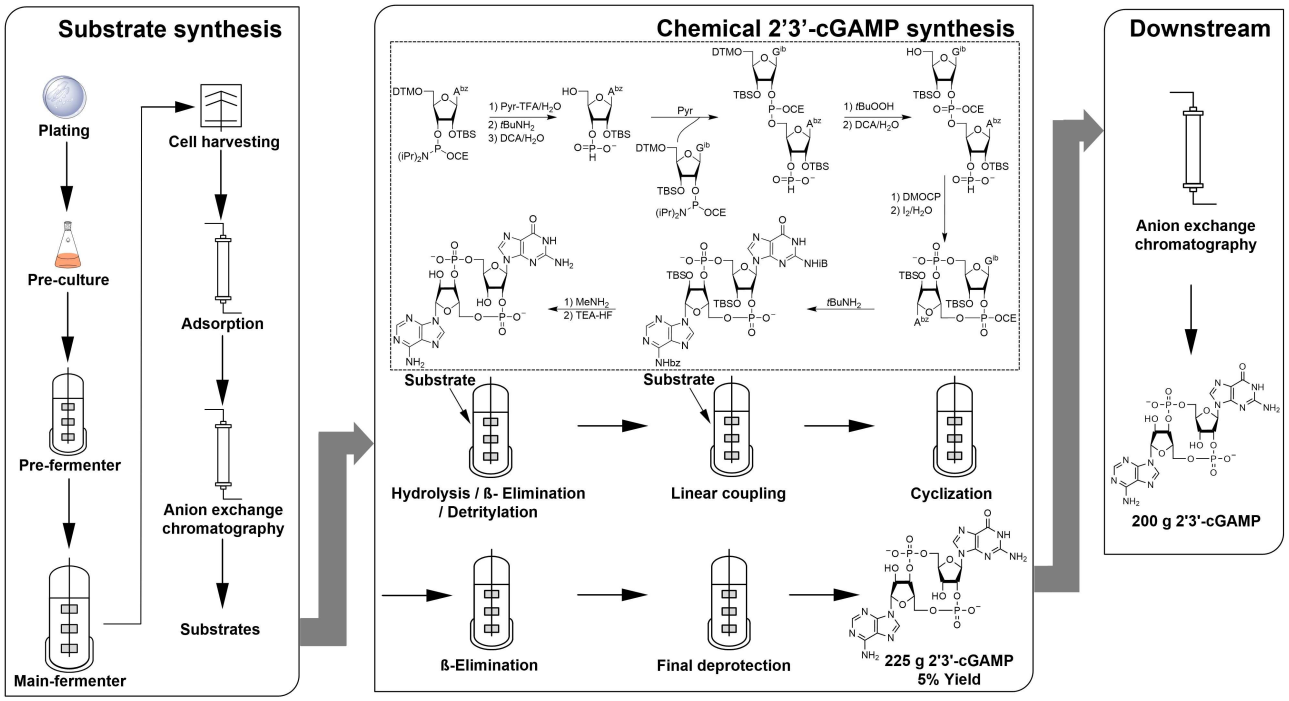

Figure 1. Process overview of the chemical synthesis of 200 g 2′3′-cGAMP. A more detailed list of all unit operations and used chemicals can be found in the Supporting Information (Table S22).

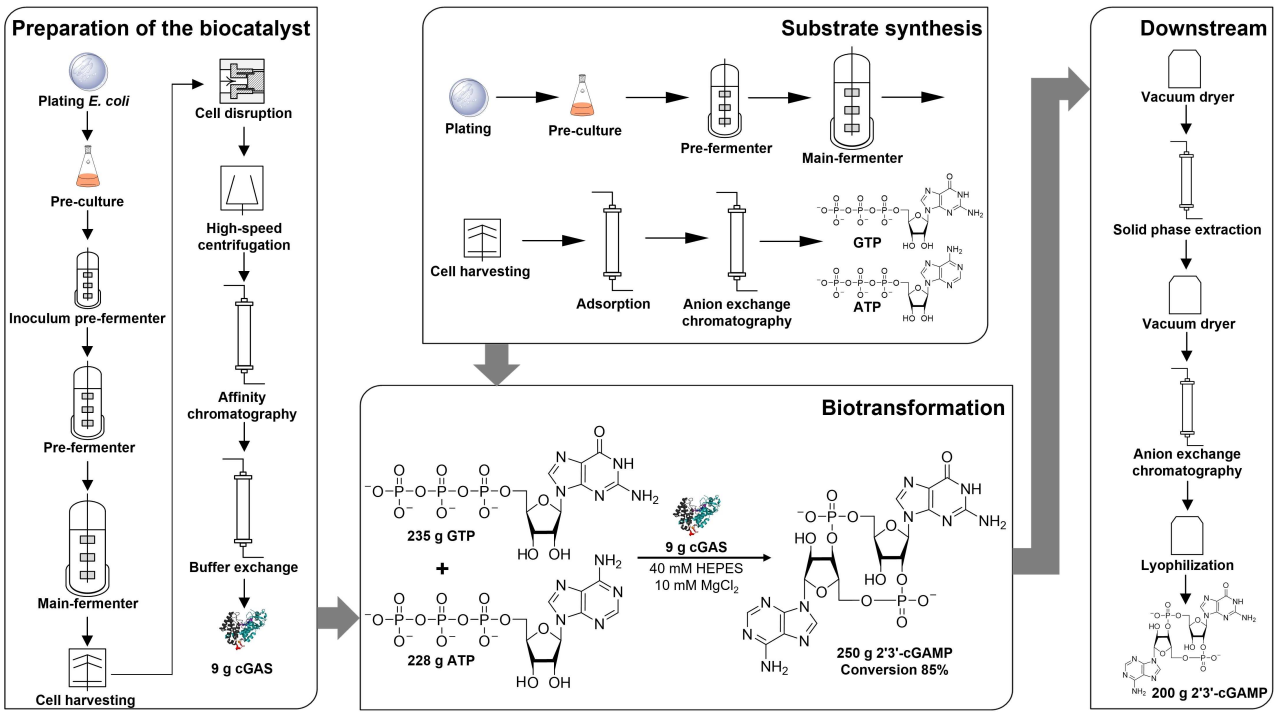

Figure 2. Process overview of the biocatalytic synthesis of 200 g 2′3′-cGAMP. The biocatalyst cGAS is prepared by an agar plate, shaking flask, two pre-fermenters, and one main fermenter. The enzyme is purified by cell disruption, affinity chromatography, and buffer exchange. The 9 g purified cGAS are used in the biotransformation, to cyclize the produced substrates ATP and GTP to 250 g 2′3′-cGAMP. It is purified by vacuum drying, solid phase extraction, anion exchange chromatography, and lyophilization.

a full life cycle inventory is not available, making a gate-to-gate LCA the most appropriate and practical approach for assessing potential environmental impacts. Gate-to-Gate is a partial LCA that allows a key unit to be identified. Therefore, the information from this methodology can be used directly to investigate technical and chemical changes to improve manufacturing processes, which are the fundamental part of the assessment. In a sensitivity analysis, the effect of solvent recycling, scale-up, energy sources of different countries as well as reusing the enzyme were investigated.

Results and Discussion

The environmental impact of the biocatalytic and chemical production route of 2′3′-cGAMP was compared using the IPCC 2021 GWP 100 and ReCiPe 2016 method. The five impact categories were used to evaluate the influence of the synthesis routes on the global warming or climate change, the environmental and human toxicity, as well as land and water use. The category "GWP" is defined over a time horizon of 100 years (GWP 100) measured in kg CO_2 equiv. and allows the comparison of the climate change impact of both routes. The "human health" category is defined by the unit disability-adjusted life years (DALYs) and thus describes the number of years of life lost and the number of years of life lost due to disability. The category "resource scarcity" considers the surplus cost of resource production in the future over an infinite period of time, taking into account a discount rate of 3% and assuming constant annual production. The category is given in USD2013. The category "ecosystems" is expressed as the loss of species over a certain area, during a certain time in years. The midpoint category "water use" is based on the amount of freshwater consumed given in m^3. Water was included in the assessment since water is used as reaction medium for biocatalytic processes resulting in diluted reaction streams, which lead to increased solvent consumption during downstream processing.[23] The five impact categories were calculated in relation to the functional unit of 200 g 2′3′-cGAMP to identify crucial parameters influencing the environmental impact of both synthesis routes.

A chemical and a biocatalytic synthesis route were considered for a comparative LCA. In addition, the downstream process for product purification was included for both synthesis routes. The biocatalytic 2′3′-cGAMP synthesis is based on the cyclization of the substrates ATP and GTP using the enzyme cGAS. For this route, the preparation of the biocatalyst cGAS, the biotransformation, as well as the downstream were considered as separate steps. The biocatalyst was considered as purified enzyme provided by heterologous expression with *Escherichia coli*. The biotransformation step included the provision of substrates and the reactions itself. A product titer of 1.15 g L^{-1} was assumed for the reaction using a substrate concentration of 2 mM and a reaction yield of 85% (based on experimental data and literature).[20] The downstream processing consisted of vacuum drying, solid-phase extraction, and

Table 1. Contribution of five environmental impact categories to the biocatalytic and chemical synthesis of 200 g 2′3′-cGAMP.[a]

Method	GWP 100 [kg CO_2 equiv.]	Human health [DALY]	Resource scarcity [USD2013]	Ecosystems [species yr]	Water use [m^3]
biocatalytic synthesis	3055.6	5.9×10^{-3}	249.0	1.4×10^{-5}	31.88
chemical synthesis	56454.0	1.1×10^{-1}	4250.1	2.7×10^{-4}	482.39
fold-difference	18	19	17	19	15

[a] GWP 100 is the 100-year time horizon global warming potential in kg CO_2 equiv. Human health indicates the disability-adjusted life years (DALYs). Resource scarcity considers the surplus cost of resource production. Ecosystems indicates the loss of species in a given period of time and in a certain area in years. Water use is given in m^3 water.

anion exchange chromatography. A product loss of 20% in two purification steps was estimated for the downstream process. The energy consumption for fermentation, biotransformation and bioreactor sterilization was calculated by extrapolating the required reactor size and calculating the energy demand of reference devices.[24,25] The energy of vacuum drying and lyophilization was calculated using general rule of thumb values.[26] The energy consumption of all other process steps was estimated using reference equipment of the appropriate scale.

The chemical synthesis consists of eight steps with *N*-benzoyl-2′-O-TBS-protected adenosine phosphoramidite as initial substrate.[21] The synthesis starts with hydrolysis, removal of the cyanoethyl group, and subsequent detritylation, whereby an H-phosphonate is obtained. The coupling with the guanosine phosphoramidite follows, which is subsequently oxidized and detritylatedforming a linear dimer. After cyclization and oxidation, the protected cyclic dinucleotide is formed. Subsequent to the extraction of 2′3′-cGAMP from the reaction mixture, the protecting group of the phosphodiester bond is removed. The final product is crystallized as triethylammonium salt and purified using anion exchange chromatography. The calculations were based on a published reaction yield of 5% for 2′3′-cGAMP, including a product loss of 10% for the downstream process (based on experimental data and literature data).[18,20]

The absolute values of the five impact categories GWP 100, human health, resource scarcity, ecosystems, and water use are summarized in Table 1 for both synthesis routes. Overall, the environmental impacts of the biocatalytic and chemical 2′3′-cGAMP synthesis differ significantly in all categories, with 15 to 19 times higher values for the chemical synthesis. For example, the GWP 100 of chemical synthesis is 56454 kg CO_2 equiv. The main reason for this large value is the low yield of only 5%.[18] The chemical route requires eight individual steps, each of which consists of various intermediate steps, which in sum leads to product loss. As no yields were published for the individual reaction steps, it is not possible to identify particularly inefficient steps, which could have served as a basis for optimizing the chemical synthesis. A few other CDNs can be synthesized by this route resulting in product yields of 17–30%,[19,27] indicating potential for optimization of the synthetic route. In comparison, the biocatalytic one-step 2′3′-cGAMP synthesis has a reaction yield of 85% generating a GWP 100 of 3055.6 kg CO_2 equiv. Hence, 2′3′-cGAMP production by biocatalytic synthesis appears to be more sustainable. In this case, the reputation of biocatalytic synthesis as often being more sustainable could be confirmed. However, this is not always the case. A recent comparative study showed that biocatalytic and chemical lactone synthesis by Baeyer–Villiger oxidation do not differ in the considered impact categories.[11] This demonstrates the importance of early LCA to support decisions on the selection of synthesis route.

In order to provide a more detailed understanding of the obtained results and to identify particularly critical steps, the percentage ratios of the process steps for the four impact categories GWP 100, human health, resource scarcity, as well as ecosystems were determined for the enzymatic (Figure 3) and chemical route (Figure 4).

The biocatalytic synthesis is divided into the biocatalyst preparation, substrate synthesis, biotransformation, and downstream processing (Figure 3). For the biocatalytic 2′3′-cGAMP synthesis, the production of the substrates has the largest contribution to the GWP 100, human health, and ecosystems, with 40–41% each. As no substance data were available in Ecoinvent for the substrates, the production data were calcu-

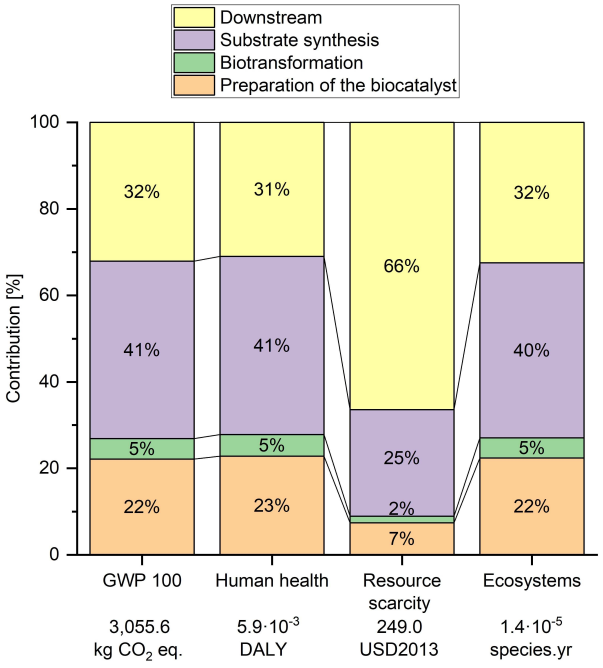

Figure 3. Comparison of GWP 100, human health, resource scarcity, and ecosystems for biological 2′3′-cGAMP synthesis for different process steps.

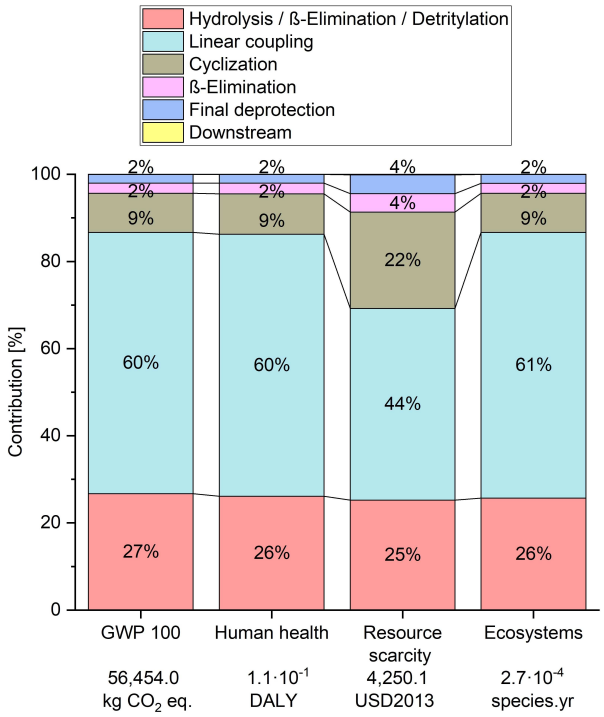

Figure 4. Comparison of GWP 100, human health, resource scarcity, and ecosystems for chemical 2′3′-cGAMP synthesis. The substrates of the chemical synthesis are not listed separately but included in the hydrolysis/β-elimination/detritylation and linear coupling step.

lated from patents.[28,29] Both substrates are fermentatively produced with an incubation time of 4–5 days and subsequently purified by adsorption on activated carbon, water reduction, anion exchange chromatography, and lyophilization (see the Supporting Information section 1.2). The long incubation time means a high energy input, which is mainly caused by the stirring of the fermenters. The energy required for cooling the bioreactors for enzyme and substrate production was neglected in the calculations. Estimations based on literature data resulted in an energy demand of 7–58 kWh,[30,31] which represents an additional demand of 0.2–1.6% relative to the total energy demand of 3590 kWh for the biocatalytic synthesis. In addition, all downstream steps are very energy-intensive, which explains the high contribution to the environmental impact categories.

The second largest contribution to GWP 100, human health, and ecosystems is accounted to the downstream with 31–32% for each impact category. The downstream processing includes vacuum drying, solid phase extraction, and anion exchange chromatography. Due to the high volume (218 L containing 1.15 g L^{-1} 2′3′-cGAMP), which leaves the biotransformation and has to be processed, the high share of the downstream on the overall process can be explained. Bioprocesses often have more complex downstream processing requirements because the use of water as a reaction medium means more sophisticated recovery.[32] Especially in this study, the product is highly diluted with a concentration of 1.15 g L^{-1}. The water has therefore to be removed in an energy-intensive manner, due to the high boiling point. The preparation of the biocatalyst cGAS is with 24% the third largest contribution in the biocatalytic 2′3′-cGAMP synthesis, which reveals a high environmental impact of the enzyme production (250 L fermenter, 9 g_{enzyme} per batch) with a GWP 100 of 75232 kg_{CO_2} kg_{enzyme}^{-1}. This value is very comparable to literature values for a purified oxidase with a GWP of 98729 kg_{CO_2} kg_{enzyme}^{-1} for a lab-scale fermentation (25 L fermenter, 1.16 g_{enzyme} per batch).[31] For a viable industrial process, the GWP for biocatalyst preparation should certainly be reduced. It can be assumed that the environmental impact per functional unit may be automatically reduced by increasing the production quantities, using a larger production scale.[31,33] For comparison, in a cradle-to-gate LCA of the production of various enzymes on industrial scale, values between 1 and 10 kg_{CO_2} kg_{enzyme}^{-1} were determined.[34,35] In the study, the purification consisted of centrifugation and filtration, resulting in crude enzyme extract that is difficult to compare with the highly purified enzyme solution used here as the basis for calculations. Nevertheless, optimizations in enzyme expression, for example, achieving higher cell densities during fermentation or higher production yields,[4,36] could be targeted to reduce the environmental footprint. Furthermore, it has to be mentioned that the biocatalyst cGAS has a low specific activity of 73 mU mg^{-1}, which causes the requirement of a large amount of enzyme for 2′3′-cGAMP production.[37] The enzyme productivities of 29 $g_{product}$ g_{enzyme}^{-1} and 2700 $mol_{product}$ mol_{enzyme}^{-1} are just and just below, respectively, the required productivities that would be necessary for economical pharmaceutical product production (calculated from Ref. [37]).[38] The basis of this calculation consists of laboratory data, which is, nevertheless, consistent with the enzymatic synthesis of other CDNs.[17] However, for an industrial application, an improved reaction rate and enzyme utilization should be aimed at, for example, by protein engineering.[39]

The contributions of the individual process steps in the case of human health and ecosystems are very similar to those of GWP 100. In the case of the impact category resource scarcity, however, there are differences. Here, the downstream accounts for 66%, the substrates for 25%, enzyme preparation for 7%, and the biotransformation for only 2% of the impact. The shift towards a higher ratio of the downstream process is due to the large quantities of required solvents for product purification.

The chemical synthesis is composed of hydrolysis/β-elimination/detritylation, linear coupling, cyclization, β-elimination, final deprotection, and downstream processing (Figure 4). The substrates were incorporated in the linear coupling and hydrolysis/β-elimination/detritylation step. As no substance data were available in Ecoinvent for the phosphoramidite substrates, they were replaced by ATP and GTP. The greatest part of the GWP 100 is accounted by the linear coupling with 60% of which 52% of the total GWP 100 is accounted by GTP used in this step. The second largest part of the GWP 100 is the hydrolysis/β-elimination/detritylation step with 26% of which 22% of the total GWP 100 is accounted by ATP used in this step. Overall, the production of the substituted substrates ATP and GTP accounted for 75% of the total GWP 100 of chemical synthesis. Since the chemical synthesis has a low yield of only

5%, larger substrate amounts are required than for the enzymatic synthesis. The cyclization of the precursor molecules contributes with 9% to the GWP 100. The β-elimination and the final deprotection each have a contribution of 2%. The downstream, which consists of an exchange chromatography, has such a small influence of 0.04% that it does not appear in the diagram. The distribution in the categories human health and ecosystems is also similar to that of GWP 100. In case of the impact category resource scarcity, the linear coupling has the biggest contribution with 44%. The hydrolysis, β-elimination and detritylation have a contribution of 25% and the cyclization 22%. The β-elimination and the final deprotection each have a contribution of 4%.

In contrast to the biocatalytic synthesis routes, the downstream process of the chemical synthesis has a comparatively low GWP 100 of 22 kg CO_2 equiv. compared to 874 kg CO_2 equiv. One reason for this is that the chemical synthesis ends in a crystallization step, which is included in the final deprotection step. Since in chemical synthesis each step involves multiple dissolving and evaporation steps to remove unreacted intermediates and solvents the final anion exchange chromatography was considered as the only downstream step. No data about product purity after chemical synthesis are available, but a sufficient product purification was assumed.

An uncertainty analysis was performed using Monte Carlo simulations with a 95% confidence level in SimaPro 9.3.02, which can be found in the Supporting Information (Table S27). A logarithmic normal distribution was assumed, and the lognormal distribution was described by standard deviation. For both synthesis routes, standard deviations between 20 and 27% were obtained in all damage categories, which typically decrease on an industrial scale.[11]

For the preparation of the analysis, some assumptions were made for the calculation. These are in particular the substitution of chemicals and the scale up of production. This could result in some changes in the results presented. Since the substrates for chemical synthesis have a very high contribution in all impact categories (e.g., 75% for the GWP 100), small changes have a strong impact on the overall result. However, since no phosphoramidites are available in the databases, no detailed estimate can be made of the effect of replacing the chemical. The production of phosphoramidites requires energy-intensive process steps such as vacuum distillation and chromatographic purification,[40,41] so it can be assumed that a high GWP 100 will still be generated by the provision of these substrates. In contrast, the contribution of water consumption will decrease, as ATP and GTP production is a very water-intensive process, unlike phosphoramidite production. Other chemicals for the chemical syntheses that were not available in the database, such as 2,5-dimethylhexane-2,5-dihydroperoxide, which is used as an initiator for linear coupling, were substituted by other chemicals with similar properties. All substituted chemicals, except of the phosphoramidite substrates, were used in amounts of 0.06–0.63% relative to the overall process. It can therefore be assumed that the effects of substitution are small, even though they were not quantifiable due to data availability.

In the case of the biocatalytic synthesis, chemicals with higher mass fractions were replaced. This includes tryptone, HEPES, and yeast extract with 5–10%, which were replaced by soy bean meal, sodium phosphate, and fodder yeast. In this case, the potential impact of the use of alternative chemicals on GWP 100 is negligible, as these chemicals represent a very small portion of the total GWP 100 of between 0.2–0.4%.

Water use

Water was considered separately, because its environmental impact is particularly relevant for enzymatic reactions, as water is usually the reaction medium. Biocatalysis is therefore considered a water-intensive process that often entails more complex downstream processing.[23,42] The water use of the two synthesis routes was calculated by the ReCiPe 2016 mid-point method. Figure 5 shows the results for the individual production steps.

The biocatalytic synthesis consumed a total of 31.9 m^3 water, while the chemical synthesis consumed 482.4 m^3 corresponding to about 15 times more water. In chemical synthesis, linear coupling accounts for the largest amount of 303 m^3 (63%) and hydrolysis of 97 m^3 (20%). This can be explained by the inclusion of substrate production in these process steps, which must be provided in high amounts due to the poor reaction yield. The cyclization requires 55 m^3, the β-elimination consumes 15 m^3 and the final deprotection 12 m^3 water. Only

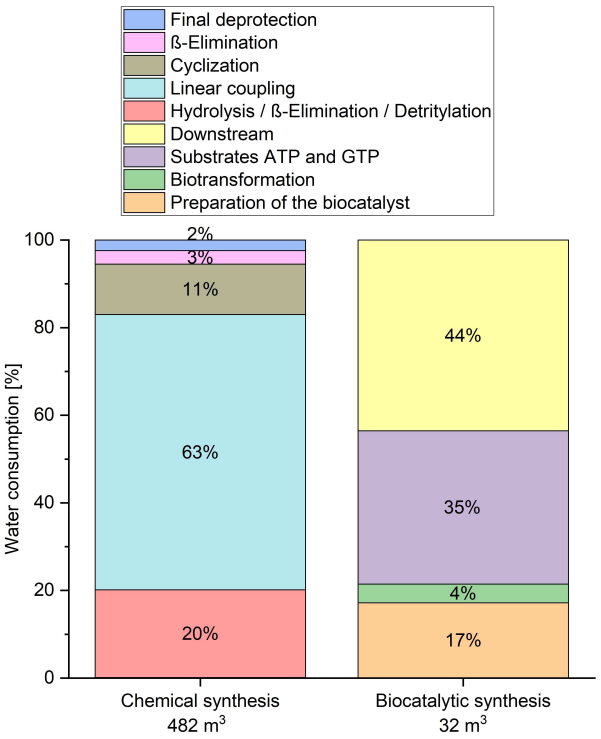

Figure 5. Comparison of water consumption in the chemical and biocatalytic 2′3′-cGAMP synthesis in different process steps. The substrates of the chemical synthesis are not listed separately but included in the hydrolysis/β-elimination/detritylation and linear coupling step.

0.5 m^3 of water is consumed in the downstream process, which is why this proportion is not visible in Figure 4.

In biocatalytic synthesis, the downstream and substrate synthesis are the most water-intensive steps, with 14 and 11 m^3 respectively. The production of the biocatalyst cGAS requires 6 m^3 of water and the biotransformation step 1.4 m^3. This shows that in biocatalytic synthesis, the substrate production also has a huge influence on water consumption of 35%.

Overall, the water intensity of the biocatalytic synthesis route still performs much better in comparison. Assuming an equal reaction yield of 85% for both synthesis routes, the water requirement of the chemical route is reduced to approximately 33 m^3, which corresponds to that of biocatalysis. The demand for water has hence not always to be worse for bioprocesses than for chemical processes, as it has also been confirmed previously in comparative studies.[11] Nevertheless, water consumption cannot be neglected, since it is significant for the bioprocess considered here (159 $kg_{water}\,g_{product}^{-1}$), even though less than for the chemical process (2412 $kg_{water}\,g_{product}^{-1}$). The water consumption of biocatalysis could certainly be reduced by process improvements. The enzyme preparation could be improved by increasing the cell density and enzyme expression level as already described in a previous study.[5] The achievable product titer could be increased by adapting the process mode or enzyme engineering as shown recently.[27] Alternative downstream processes could further decrease the water consumption. For example, a simplified 2′3′-cGAMP purification has been demonstrated by acidifying of the solution, which gave quantitative precipitation.[27] Although it is not yet possible to predict the applicability of this method and the product purity that can be achieved, these studies show that there is still a great potential for optimization.

Effect of solvent recycling in chemical synthesis

Solvent consumption accounts for more than 60% of total mass consumption in the pharmaceutical industry.[43] A significant number of solvents have a negative impact on the environmental sustainability of products due to health, fire, and explosion hazards. The production of solvents is often based on fossil raw materials and their disposal is complex and costly. For the chemical 2′3′-cGAMP synthesis, a total of 3740 L of the solvents ethyl acetate, diethyl ether, acetonitrile, dichloromethane, methanol, pyridines, ethanol, acetones, methylamine, and triethylamine are consumed. The solvents ethyl acetate, diethyl ether, acetonitrile, and dichloromethane account for about 2400 L. In comparison, biocatalytic synthesis requires only about 335 L of solvents. Due to the relatively low solvent consumption of the biocatalytic 2′3′-cGAMP synthesis, the effects of solvent recycling were not evaluated here.

As a possible optimization of the chemical 2′3′-cGAMP production, the impact of solvent recycling of the four main contributing solvents on GWP 100 was calculated assuming a recycling efficiency of 90%. The energy required for the recycling process was not considered, as it is assumed that the solvent is already recovered from the process in a reasonably pure form. Acetonitrile is mainly used for the first synthesis step (hydrolysis/β-elimination/detritylation) and the second step (β-elimination). Dichloromethane is predominantly used for hydrolysis/β-elimination/detritylation and linear coupling. Dichloromethane and diethyl ether are used for cyclization. Figure 6 shows the results dependent on the individual process steps.

Solvent recycling reduces the overall GWP 100 from 56454 to 49599 kg CO_2 equiv., which represents a reduction of 12%. Cyclization has the largest savings of 60% with a total of 3036 kg CO_2 equiv., followed by hydrolysis/β-elimination/detritylation and linear coupling with 1769 and 1240 kg CO_2 equiv., respectively. The solvents dichloromethane and diethyl ether have particularly negative environmental impacts, safety issues and require complicated disposal.[44] It is therefore preferable to apply such recycling strategies. Acetonitrile is one of the less problematic aprotic solvents, and ethyl acetate has low negative environmental impacts.[44]

Effect of electricity source

One of the largest polluters in the world is the power sector with 69% of global greenhouse gas emissions.[45] The type of energy production has a significant impact on the emission of greenhouse gases. The composition of the energy mix differs depending on the considered country, which is reflected in different GWP 100 per kWh. For the calculation of the GWP 100 of the two synthesis routes, the German medium-voltage market was used in SimaPro. To investigate the impact of energy generation on both 2′3′-cGAMP synthesis routes, GWP 100 was also calculated for the global market, Norway, and Europe (Figure S1).

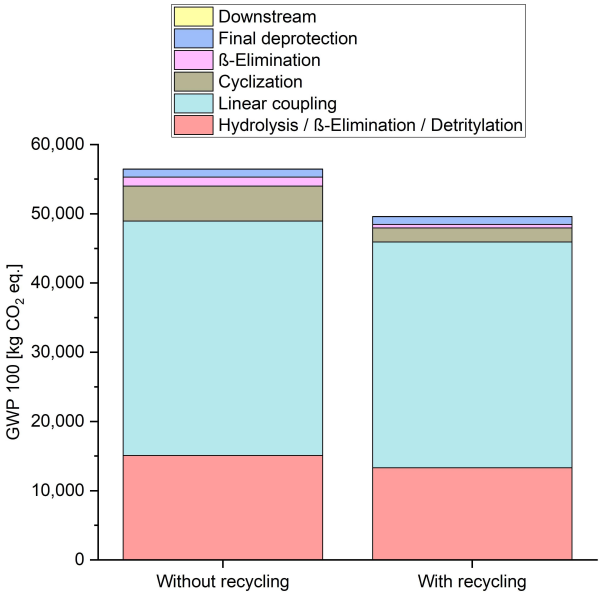

Figure 6. Effect of 90% recycling of the solvents (acetonitrile, dichloromethane, diethyl ether, and ethyl acetate) in the chemical synthesis on the GWP 100.

The energy mix in Germany consists mainly of coal and natural gas, while in Norway it is generated mainly from hydropower. In total, the GWP 100 for the biocatalytic 2′3′-cGAMP synthesis in Germany is 3056 kg CO_2 equiv., of which 20% of is caused by energy production. In Norway, 1152 kg CO_2 equiv. are emitted, which is only 38% compared to the GWP 100 of Germany. Globally, the GWP 100 is 15% higher and in Europe, 20% lower compared to Germany. Chemical 2′3′-cGAMP synthesis causes 56454.0 kg CO_2 equiv. in Germany, of which 5% is due to energy consumption. The selection of the respective electricity source has exactly the same effects on the chemical process as on the biocatalytic process.

These results demonstrate that the geographical location substantially contributes to the GWP.

Effect of enzyme immobilization

The biocatalytic synthesis was also investigated with regard to possible optimizations. A frequently used approach to reduce costs of a process is the reuse of the biocatalyst through immobilization.[46] Immobilization can increase the stability of the enzyme.[47] At the same time, immobilized enzymes can be more easily separated from the product solution and fed to a new batch. Thus, it is not necessary to produce and purify the enzyme for each batch, which saves laborious process steps and simplifies downstream processing. Since no data are yet available on the influence of cGAS immobilization on yield and stability over several reuse cycles, different cases were calculated. Either 0, 2, 5, or 10% yield loss after each batch was assumed. The calculations refer to the impact of reuse on the GWP 100 to produce 200 g 2′3′-cGAMP. Figure 7 shows the impact of cGAS immobilization in the biocatalytic synthesis for different scenarios of enzyme reuse.

In the first cycle, the previously calculated GWP 100 of 3056 kg CO_2 equiv. was calculated, which changes in dependence of the cycles of reuse and on the yield loss. The GWP 100 converges from 3056 kg CO_2 equiv. to a continuum of about 2420 kg CO_2 equiv. after 10 to 15 batches, which is a reduction of 20%. With a 2% yield loss per batch, the GWP 100 decreases to 2618 kg CO_2 equiv. within the first 5 cycles and increases thereafter. After 23 cycles of reuse, the GWP 100 exceeds the 3056 kg CO_2 equiv. and thus the initial value without immobilization, so that further reuse of the enzyme is no longer beneficial in terms of GWP. The situation is similar for the 5 and 10% yield losses after immobilization. In the case of the 5% yield loss, the global warming potential exceeds 3056 kg CO_2 equiv. after the 9th reuse and in the case of the 10% already after the fifth reuse.

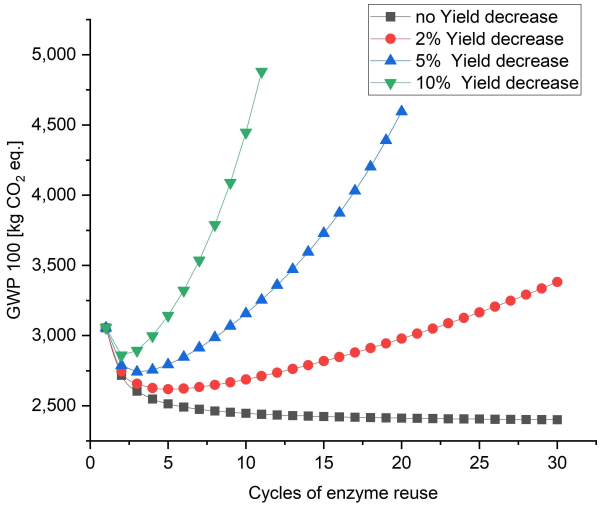

Figure 7. Influence of enzyme immobilization of cGAS for the production of 200 g 2′3′-cGAMP on GWP 100 as a function of reuse.

These results demonstrate that enzyme immobilization and reuse can be beneficial for 2′3′-cGAMP synthesis. However, this advantage can only be achieved if the enzyme is stable without significant loss of activity. It is necessary to mention that no calculations were made about the influence of the immobilization process itself on the GWP 100. In further considerations, the impact of immobilization should also be calculated in order to estimate whether a reduction of GWP can be achieved.

Effect of scale-up

To investigate the impact of the production scale, the influence of a scale up on GWP 100 was roughly estimated for both synthesis routes. The contribution of the chemicals used was extrapolated for the synthesis of 100 kg 2′3′-cGAMP and amounted 647531 kg CO_2 equiv. for the biocatalytic and 8580199 kg CO_2 equiv. for the chemical synthesis route (Table S26 in the Supporting Information). The trend of the three most influential energy demands stirring, sterilization and vacuum drying, which together account for more than 90% of the total energy demand in both routes, was estimated.

An energy saving of 23% per kg product can be assumed for stirring, which was calculated based on reference devices.[24] Based on published data,[31] the energy demand for sterilization was estimated, which amounted to an energy saving of 89% per kg product. The energy required for the evaporation of aqueous solutions can be generally estimated at 600–700 kWh m^{-3} water.[48] If heat integration is assumed,[49] as is the case with large-scale processes, a reduction to approximately 17 kWh m^{-3} water can be expected resulting in an energy saving of 97%. For chemical synthesis, the same scaling values are assumed for the evaporation steps, even though some reaction steps use organic solvents rather than water, which require a different energy input depending on the solvent. The overall reduction of energy required per kg product led to a reduction of 26% kg CO_2 equiv. per kg product for both synthesis route.

The reduction of the environmental impact per kg has already been reported for increasing the production scale of other products. For example, in the enzymatic production of nanocellulose yarn from carrot waste, the impact was reduced by a factor of up to 6.5 compared to the laboratory production.[50] The main reason for this large difference is that

when the laboratory scale is transferred to the industrial scale, many devices are no longer comparable, and heat and energy recovery are often not taken into account.

Conclusion

In this study, a comparative life cycle assessment (LCA) of the biocatalytic and chemical 2′3′-cGAMP synthesis was performed at an early stage of development based on laboratory as well as literature data. Due to the complexity as well as the low yield of the chemical synthesis and, on the other hand, the low titer of biocatalytic synthesis, a simple estimation based on these key parameters was not possible. A global warming potential (GWP) 100 of 3055.6 kg CO_2 equiv. was determined for biocatalytic synthesis and 56454.0 kg CO_2 equiv. for chemical synthesis, which is 18 times higher. This trend was also observed in the other impact categories. Therefore, based on the LCA results, the biocatalytic 2′3′-cGAMP synthesis was identified as a significantly more environmentally friendly route. This proves that an early LCA on the basis of laboratory data and approximations is suitable for identifying the most environmentally friendly synthesis route. Furthermore, it allows to identify inefficient synthesis steps and to evaluate their optimization. In case of 2′3′-cGAMP synthesis, solvent recycling in chemical synthesis and enzyme reuse in biocatalytic synthesis were examined in detail with regard to possible improvements. A GWP reduction of 12% was achieved through solvent recycling for the chemical synthesis and up to 20% through enzyme reuse for the biocatalytic synthesis. The importance of different energy sources and the composition of the energy mix were shown.

An early assessment of the environmental impact of different synthesis routes can be particularly useful at an early development stage of the manufacturing processes for new products. However, the preparation of an LCA is complex and requires special expertise. In the case of biocatalytic LCAs, limited availability of substance data in LCA databases complicates the preparation of the life cycle inventory analysis. For the present study, separate LCAs were generated for the substrates yeast extract, adenosine 5′-triphosphate (ATP) and guanosine 5′-triphosphate (GTP). Several other substrates had to be replaced by chemically similar substrates. In the future, it would be desirable to adapt databases for the environmental assessment of bioprocesses in order to simplify the preparation of LCAs and to contribute to the development of more sustainable production processes.

Experimental Section

This LCA was prepared according to ISO standard 14044:2006. The synthetic routes were modeled in SimaPro 9.2 (PRé Sustainability B.V., NL) using the Ecoinvent 3.7.1 database (Ecoinvent Center, St-Gallen, Switzerland). Furthermore, the production of the chemicals ATP, GTP, and yeast extract required as substrates for the reaction step and required as growth medium, respectively, were modeled and included in the calculation. Environmental impacts were calculated with the ReCiPe 2016 and IPPC 2021 GWP 100 method.

Goal and functional unit

The aim of this study was a comparative LCA between biocatalytic and chemical synthesis of 2′3′-cGAMP. For both syntheses, data were extrapolated from the laboratory scale for synthesis and purification of the product 2′3′-cGAMP. A functional unit (FU) of 200 g 2′3′-cGAMP was chosen as product amount, which is representative for the range required for example, preclinical or phase 1 clinical studies.[22]

System description and boundaries

The boundaries of this comparative LCA are gate-to-gate considering the synthesis and purification of the product 2′3′-cGAMP, which is synthesized either chemically from phosphoramidites, or enzymatically from ATP and GTP using cGAS as biocatalyst.

The biocatalytic 2′3′-cGAMP synthesis (Figure 2) was calculated based on a detailed synthesis protocol at lab-scale for cGAS production[20] with 2 mM substrate concentration and 85% conversion in the biotransformation. The LCA of biocatalytic 2′3′-cGAMP synthesis includes the production of the nucleoside triphosphate substrates and yeast extract, enzyme production and preparation, biotransformation, and purification of the final product 2′3′-cGAMP. For all incubation steps, initial sterilization of media and equipment was considered. The energy consumption for incubation was considered as well as for stirring, which was calculated according to the volume on the basis of extrapolation of reference reactors.[24] The amount of cooling water required for the fermenters in addition to the water, energy, and detergent consumption for cleaning of all the devices were not considered. Since no information was available on the substrates and yeast extract in Ecoinvent, these were calculated and used for further modeling. The substrates ATP and GTP are produced by fermentation process. The calculated production of 228 g ATP is based on a patent using the fermentation strain *Methylomonas probus*.[28] The production includes pre-cultivation steps (agar plate, shaking flask, and 1 L pre-fermenter) and the main production step in a 50 L fermenter (26 L working volume, 96 h) using diammonium phosphate and methanol as growth substrates. In addition, the production of 235 g GTP was calculated based on a patent using the fermentation strain *Brevibacterium ammoniagenes*.[29] The pre-cultivation strategy was adopted from ATP production. The main production step consists of a 145 L fermentation in a 250 L fermenter for 120 h using glucose, peptone, and yeast extract as growth substrates. The purification of both nucleoside triphosphate substrates consists of cell separation, adsorption on activated carbon, water reduction by vacuum drying, anion exchange chromatography, and product lyophilization. The production of the 3.1 kg yeast extract, which is required for the production of the biocatalyst cGAS was calculated based on a patent using *Saccharomyces cerevisiae*.[51] The strain was pre-cultivated using agar plate, shaking flask and pre-incubator. The main fermentation consists of a 230 L incubation in a 350 L fermenter for 20 h using corn steep liquor as energy source. The purification contains cell harvesting, washing, autolysis, and vacuum drying of the final product.

For biocatalyst production, the expression strain *E. coli* BL21 (DE3) pLysS pET28a-*SUMOthscGAS* was pre-cultivated using agar plate, shaking flask, and two pre-fermenters (3 and 30 L). The expression was performed in with a 275 L fermentation (400 L fermenter, 14 h) with tryptone and yeast extract as growth medium. Cells were harvested by a disc separator, resuspended in lysis buffer, and

disrupted by a high-pressure homogenizer. After removal of cell debris by a high-speed centrifugation, the enzyme cGAS was purified by immobilized metal affinity chromatography using Ni Sepharose® 6 Fast Flow as stationary phase. Gel filtration using Sephadex G-25 medium was used to remove interfering imidazole. An amount of 9 g purified enzyme was used for the biotransformation. The biocatalytic 2′3′-cGAMP synthesis was performed with substrate concentrations of 2 mM ATP and GTP at 37 °C with a yield of 85 %, which was confirmed in laboratory experiments at a 1 mL scale. The reaction volume is 218 L and incubated for 24 h in a 225 L reactor. The product 2′3′-cGAMP is subsequently purified in order to obtain 200 g 2′3′-cGAMP by the following process steps; water reduction by vacuum drying, solid phase extraction, anion exchange chromatography, and lyophilization. The detailed mass and volume flows, as well as chemicals and energy used, can be found in the Supporting Information.

The chemical 2′3′-cGAMP synthesis (Figure 1) was calculated based on a detailed synthesis protocol with a yield of 5 %.[18,21] The synthesis contains eight main steps, which in turn can be divided into 20 sub-steps. Due to the complexity of chemical synthesis, only the major steps are explained here. A detailed list can be found in the Supporting Information (Table S22). As no substance data were available for the *N*-isobutyryl-2′-*O*-TBS-protected educts adenosine phosphoramidite and guanosine phosphoramidite, they were replaced with the corresponding nucleotides ATP and GTP. All evaporation steps were replaced by 60 % filled vacuum dryers. The synthesis starts with drying of the guanosine phosphoramidite twice with acetonitrile in a vacuum reactor. In the next synthesis step, the hydrolysis of the adenosine phosphoramidite follows by removal of the cyanoethyl group, and subsequent detritylation, whereby an H-phosphonate is obtained. Afterwards, the H-phosphonate is coupled with the dried guanosine phosphoramidite. The formed linear dimer is subsequently oxidized and detritylated. Subsequently, the cyclization of the linear dimer takes place and is oxidized. Then, the extraction of the protected CDN from the reaction mixture is carried out. After the protecting group on the phosphodiester bond is removed, the final product 2′3′-cGAMP is crystallized as triethylammonium salt. It is purified in the downstream using anion exchange chromatography and lyophilization. The detailed mass and volume flows, as well as chemicals and energy used, can be found in the Supporting Information.

Used data sources

The data for the LCA come from primary laboratory data, literature references and own estimates (Table S28). They are divided in order of importance in several categories:

Primary data: Laboratory-scale experiments were used and extrapolated. This concerns experiments for the preparation of the biocatalyst cGAS and the biocatalytic synthesis as well as purification of 2′3′-cGAMP, which were performed in our group.[20,37]

Secondary data: Extrapolated literature data were used to model the chemical synthesis and purification of 2′3′-cGAMP as well as the synthesis of ATP, GTP, and yeast extract.[18,21,28,29,52] The literature data were based on gram-scale.

Secondary data from the Ecoinvent 3.7.1 database (Ecoinvent Center, St-Gallen, Switzerland) were used for all available basic chemicals. The data for the electricity production were obtained for the global, European, and Norway market.

Data from alternative chemicals were used for chemicals that were not available in the Ecoinvent database. The alternative chemicals have equivalent functions. In the case of biocatalytic 2′3′-cGAMP synthesis, this refers to: tryptone: soy bean meal; yeast extract: fodder yeast; HEPES buffer: sodium phosphate; potassium phosphate: sodium phosphate; diammonium phosphate: ammonium sulfate; peptone: fodder yeast; guanine: imidazole and magnesium chloride hexahydrate: calcium chloride. In the case of chemical 2′3′-cGAMP synthesis this refers to: adenosine phosphoramidite: ATP; guanosine phosphoramidite: GTP; *tert*-butyl hydroperoxide: 2,5-dimethylhexane-2,5-dihydroperoxide; dichloroacetic acid: chloroacetic acid; decane: heptane; DMOCP: acetyl chloride.

Suppressed data: For chemicals used in small quantities, a cut-off rule of 0.01 % was applied. For chemical synthesis this concerns 55 g argon (0.000005 %). For biocatalytic synthesis, this concerns 153.35 g imidazole (0.009 %), 135.6 g TRIS-HCl (0.008 %), 32.8 g IPTG (0.002 %), 22.5 g HT-DNA (0.001 %), 13.9 g kanamycin (0.0008 %), 4.3 g TCEP (0.0003 %), 3.3 g chloramphenicol (0.0002 %), and 1.5 g Agar (0.00009 %).

Data quality

An uncertainty analysis was performed using Monte Carlo simulations with a 95 % confidence level in SimaPro 9.3.02 for both synthesis routes. To quantify the uncertainties, it was assumed that the measurement data have a logarithmic normal distribution in the LCA, and the lognormal distribution is described by a standard deviation (SD). 1000 Iterations were performed to derive measures of uncertainty for the chosen environmental damage categories (Table S27 in the Supporting Information).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: biocatalysis · cyclic dinucleotides · enzyme catalysis · life cycle assessment · sustainable chemistry

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