Scientific contribution/Original research

Impact of a Saccharin Higher Homolog on *Saccharomyces cerevisiae*

Gabrovšek A[1], Tašler N[1], Barrios-Francisco R[2], Jeran M[3,4,5,*]

1. University of Ljubljana, Biotechnical Faculty, Ljubljana, Slovenia
2. Tecnológico Nacional de México/TES de San Felipe del Progreso, División Ingeniería Química, San Felipe del Progreso, Mexico
3. University of Ljubljana, Faculty of Health Sciences, Laboratory of Clinical Biophysics, Ljubljana, Slovenia
4. University of Ljubljana, Faculty of Electrical Engineering, Laboratory of Physics, Ljubljana, Slovenia
5. "Jožef Stefan" Institute, Department of Inorganic Chemistry and Technology, Ljubljana, Slovenia

* Correspondence: Marko Jeran; marko.jeran@ijs.si

Abstract:

Saccharin is an organic compound, which is often used as a calorie-free artificial sweetener. It salts are being produced for the market for over 80 years. Saccharin and its derivates are very applicatory oriented, therefore researchers synthesize more and more active ingredients, which could potentially show better performance.

This work considers the effect of biological activity of a newly synthesized saccharin derivative Methyl 4-hydroxy-1,1-dioxo-2*H*-1,2-benzothiazine-3-carboxylate (6Sac) on yeast *Saccharomyces cerevisiae*. Qualitative comparison of the studied activity with the activity of the saccharine sodium salt is presented. Our results were gained by two different ways of viability detection: counting dead/live cells dyed with methylene blue and counting colony-forming units (CFU). The study has shown that the saccharine derivative with an ester functional group has negative effect on growth and reproduction of yeast. The qualitative comparison of the activity of the tested substance with the already known activity of saccharine sodium salt is a convenient method for following the model organism *Saccharomyces cerevisiae*.

Keywords: Saccharin, sodium saccharinate, *Saccharomyces cerevisiae*, Viability, Methylene blue, Colony-forming units (CFU), Medicine

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1. Introduction

Calorie-free synthetic sweeteners are commonplace in the food industry and kitchens. Nonetheless, health issues may arise following their frequent ingestion (Ager et al, 1998). In fact, carcinogenicity studies thereof, did not reveal any tangible deleterious impact on human health, by contrast to the bladder cancer which resulted in rats (Weihrauch et al., 2004). In particular, saccharin which means "similar to sugar" (labelled E954) (Božič, 2016) is a common zero-calorie organic sweetener (Namartha et al., 2016). It is 200–700 times sweeter than saccharose (table sugar) but with a mild bitter metallic taste. It has been commercially available for >80 years (Report on carcinogens, 1998) following its accidental discovery in 1878 by the chemist Constantin Fahlberg during his work on coal-tar (Namartha et al., 2016). Saccharin is thermally stable at food baking temperatures and is inert towards other ingredients at room temperature. Structurally, saccharin belongs to the benzosultam class of organics; notably *gamma*-sultam for this 5-membered ring compound (Mihali, 2012). Owing to its relative *N*-H acidity (pK_a 2.32; with low water solubility), it can readily form salts and is also available as its highly water-soluble sodium or calcium salt (as white powders) (Namartha et al., 2016).

Antibiotic-resistant bacteria present a serious general health problem inciting the research and study of new heterocyclic compounds – *e.g.* the ones containing sulfur and nitrogen atoms–which are known to offer a wide spectrum of bioactivity (Patel et al., 2016). In addition, the diversification of a given basic molecule by appending different functional groups onto it may fortuitously endow a new derivative with an interesting bioactivity (Mehta, 1961).

2. Literature studies on the bioactivity of saccharin and its derivatives

Studies have shown that saccharin may cause cancer in animals qualifying it in 1981 to the list of carcinogens (Report on carcinogens, 1998). Later, many researchers have investigated the health impact of saccharin and its derivatives.

In a study wherein lab rats were fed a high level of 5% of various saccharin compounds out of 20 screened showed a significant increase of neoplasia. In a two-generation study, parents and offsprings were put on a saccharin diet noticing that the latter had developed an increased risk of bladder cancer (Weihrauch et al., 2004). Similar results were obtained switching to sodium saccharinate. It was indicated that these compounds impact the urinary system physiology; this concerns urine pH, osmolarity, volume, and the appearance of a precipitate. In another study on mice, saccharin was found to affect the structure of the microbiome by impairing glucose homeostasis (Spanogiannopoulos et al., 2016). As saccharin impact on monkeys or humans did not show enough evidence for increase of cancer risk, saccharin was de-listed from the carcinogens list (Weihrauch et al., 2004). In the presence of *S. cerevisiae* saccharin was found to inhibit the sporulation process and slightly increase the occurrence of diploid and disomic meiotic products without affecting the frequency of recombination (Persic, 1986). At a concentrations of 2–20 mg/mL, sodium saccharinate did not significantly affect cell growth, but slightly reduced cells survival; cell survival decreased even more at a concentration of 100 mg/mL (Moore et al., 1979).

Various benzosultams to which class saccharin belongs have interesting bioactivity and are therefore used in general as pharmaceuticals (Mihali, 2012). For example, a series of Schiff's base derived from *N*-substituted saccharin showed activity against some microorganisms (Musa, 2016). These displayed an interesting inhibition of cholinesterase which is potentially applicable to treating Alzheimer's disease (Hebda et al., 2016). Moreover, it was indicated that 1,2-benzothiazines possess anti-inflammatory, antidepressant, antimicrobial, and anti-carcinogenic properties such as the saccharin-derived anti-inflammatory agents piroxicam, ampiroxicam, and meloxicam (Patel et al., 2016). Screening 45 compounds of 1,2-benzothiazine, none of them showed any activity against Gram-negative bacteria (*i.e. Proteus vulgaris* and *Salmonella typhimurium*), but 12 compounds showed activity against Gram-positive bacteria (*i.e. Bacillus subtilis* and *Staphylococcus aureus*) (Patel et al., 2016). Finally, in a study concerning the inhibitory activity of saccharin-based compounds on the interferon signaling pathway, a compound was identified for possible therapeutic use. It inhibited the lipopolysaccharide signaling in primary macrophages without showing any cytotoxicity at concentrations

up to 100 μM. This could be potentially useful for therapy in inflammatory diseases such as systemic lupus erythematosus and multiple sclerosis (Csakai et al., 2014).

3. The goal and hypothesis

This work concerns the bioactivity of saccharin higher homolog 6Sac (**Figure 1**) against *Saccharomyces cerevisiae* (*S. cerevisiae*). Methyl 4-hydroxy-1,1-dioxo-2*H*-1,2-benzothiazine-3-carboxylate (6Sac), a saccharin higher homolog, which possesses a functionalized 6-membered ring (*i.e.* a *delta*-sultam instead of a *gamma*-sultam). 6Sac has been obtained by synthesis starting from saccharin by cycle enlargement/extension (**Figure 1**) (Jeran et al., 2017).

To observe the saccharin higher homolog 6Sac (**Figure 1**), we used two different methods – measurements of colony forming units (CFU) and determination of viability with methylene blue dye. We anticipated that compound 6Sac will show fungistatic and fungicidal activity against *Saccharomyces-cerevisiae* (*S. cerevisiae*).

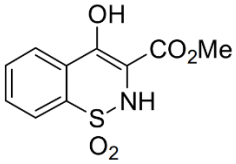

Figure 1: Structure of Methyl 4-hydroxy-1,1-dioxo-2*H*-1,2-benzothiazine-3-carboxylate (6Sac).

4. Methods

4.1. Culturing

To the liquid medium (*see below*) was added the inoculum (yeast containing active *S. cerevisiae*), and left undisturbed for 3 days. The suspension was diluted and divided into eight Erlenmeyer flasks. Two flasks served as controls: to one flask was added dimethyl sulfoxide (DMSO), and another one was kept unchanged. To the remaining six flasks, the test substance 6Sac was added. After 5 hours the cells were counted then recounted after every 20 hours for the next three days.

Two different methods for viability determination were applied: cells were grown on hard agar medium which permits colony-forming units (CFU) count, and viable cells were counted under Bürker-Türk chamber following addition of methylene blue dye.

The statistical analysis was performed by Microsoft Excel (Microsoft Corporation, version 2018). The mean values and the standard deviations of the two populations (treated and untreated samples) were calculated. The statistical significance of the differences between the two populations were calculated by the two-tailed *t*-test. The values of the probability *p* smaller than 0.05 were considered as statistically significant.

4.2. Preparation of the liquid medium (Biolife)

The reagents (*5.00 g of yeast extract* (Spar), anhydrous D-(+)-glucose (10.0 g) (*Sigma-Aldrich*), mixed powder extract (Torlak) (10.0 g)) were weighed into an Erlenmeyer flask and distilled water (200 mL) was added. The suspension was transferred to a 500 mL flask and distilled water added to reach the 500 mL level. The contents of the flask were mixed well and poured into the Erlenmeyer flask. The flask was heated on a burner to boiling till a clear solution was obtained, then covered with aluminium foil and kept for 20 min at 121 °C in an autoclave.

4.3.Preparation of the YGC agar solid medium

The powder medium contained 14.9 g/L YGC agar (Sigma-Aldrich, 9576), 0.1 g/L chloramphenicol, 20 g/L D-(+)-glucose, and 5 g/L yeast extract. To prepare 500 mL of a medium, Erlenmeyer flask was weighed with 20.0 g of YGC agar, and the contents to the 500 mL mark diluted with distilled water.

The flask was heated on a burner to boiling till a clear solution was obtained. It was then covered with aluminium foil and kept for 20 min at 121 °C in an autoclave. Then, the flask was placed at 45 °C in a water-bath for the mixture not to harden. When it cooled down, it was poured into petri dishes.

4.4. The growth curve

250 mL of liquid medium was inserted in an Erlenmeyer flask. After autoclaving and cooling the sample, 0.5 g of fresh yeast *S. cerevisiae* (Fala) was inoculated to the medium.

The procedure was performed under sterile conditions. The medium with *S. cerevisiae* was put on a shaker, so it was constantly mixing to obtain homogeneous broth with enough oxygen uptake. We took 1 mL of broth out of Erlenmeyer flask at 0, 22, 44, 66 and 144 hours and we made different dilutions of sample in 0.9% NaCl solution (countability, under microscope). Dilution of the sample was applied to the Bürker-Türk counting chamber, so we were able to count cells under a microscope and calculate the concentration of cells in broth. Concentration told us, when our cells are in stationary growth phase, what we used later in experiment.

4.5. Effect of compound 6Sac on S. cerevisiae

For first stage, we prepared a mixture of 125 mL liquid medium (described in chapter 5.1) in a 500 mL Erlenmeyer flask and then added 0.250 g of *S. cerevisiae*. The contents were stirred on a magnetic stirrer at room temperature for two days, so our culture came into the stationary phase of growth. After two days it was used as 25% inoculum for second stage, so we diluted our 125 mL of broth with 375 mL of the fresh liquid medium (the same one as before, described in chapter 5.1), stir it, so it was well homogenised and used it as fresh broth for second stage.

For second stage, we used eight 100 mL Erlenmeyer flasks. Firstly, we prepared empty Erlenmeyer flasks with compounds and then we added fresh broth for second stage. In first three Erlenmeyer flasks were added 50 mg of compound 6Sac and 500 μL of DMSO (1.0 mg/mL 6Sac + DMSO). The contents were mixed well to dissolve the 6Sac completely. We repeated the process for the next three Erlenmeyer flasks, where we added 500 mg of 6Sac and 500 μL of DMSO (10.0 mg/mL 6Sac + DMSO). In seventh Erlenmeyer flask we put just 500 μL of DMSO (Control) and eighth Erlenmeyer flask remained empty at that point (Control, blank). Then we divided our fresh broth for second stage in all eight Erlenmeyer flasks – we added 50 mL in each. All Erlenmeyer flasks were covered with aluminium foil and marked accordingly. They were placed on shaker and shaked constantly at room temperature for next three days.

After 5, 25, 45 and 65 hours, we aseptically took 1.00 mL of sample from all eight Erlenmeyer flasks ad we prepared different dilutions in 0.9% NaCl solution. Hundred-fold diluted samples were mixed with methylene blue solution in a volume ratio of 1:1. Methylene blue was left to act for one minute. The contents were mixed well with the vortex mixer so that the cells were evenly distributed. The sample was cannulated onto the Bürker-Türk counting chamber, covered with a cover glass, and mounted to a microscope for counting the total number of cells and calculating the concentration of cells in broth. We also counted the number of living and the dead cells, because dead cells remained blue coloured, due to methylene blue.

All eight samples were also inoculated on plates with solid medium (described in chapter 5.2). We used a 10^{-5} and 10^{-6} dilution of a samples. 100 μL of the dilution was applied to the solid medium under sterile conditions and spread over by using a Drigalski spatula. Both dilutions of all eight samples were plated on 3 plates. All plates with solid medium were incubated for 24 hours at 37 °C. Due to the incubation process and potential infections (contamination), blank sample and solid medium without applicable solutions tests were also performed. The CFU formed were counted after 24 hours.

5. Results and Discussion

5.1. *S. cerevisiae growth curve*

The optimum time window for the growth and activity of the *S. cerevisiae* cells was between 44–66 h, which translates to *i.e.* 2–3 days (**Figure 2**).

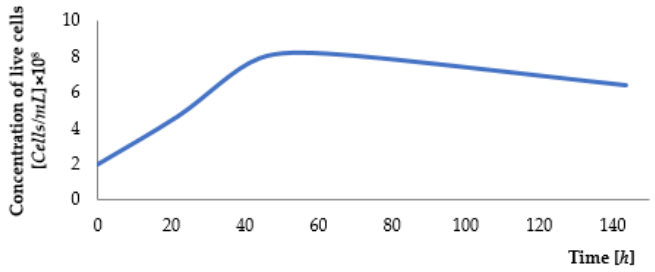

Figure 2: *S. cerevisiae* growth curve.

5.2. *S. cerevisiae cultiviability in presence of substance 6Sac*

In the results of counting CFU on solid medium plates, we found that the addition of 6Sac to the yeast suspension successfully lowered the yeast growth as the cell concentration did not exceed 2 CFU/mL × 10^7 (**Figure 3**).

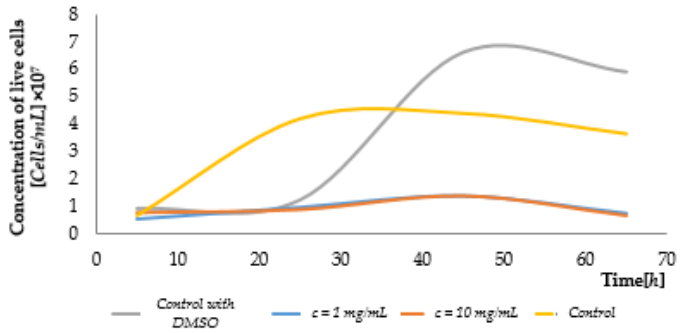

Figure 3: Concentration of live *S. cerevisiae* cells in presence of 6Sac from CFU results.

5.3. *Viability of S. cerevisiae in presence of 6Sac.*

We also got results about concentration of live cells with counting under microscope and we can see them in **Figure 4**. In the presence of 6Sac, concentration of live *S. cerevisiae* cells increased for about 45 h and then it started to decrease except for the control with DMSO (**Figure 4**). We can see that DMSO did not have a large impact on the concentration of live cells, if we compare control and control with DMSO. We can also see that there was no significant difference in the number of live cells between both concentrations of 6Sac.

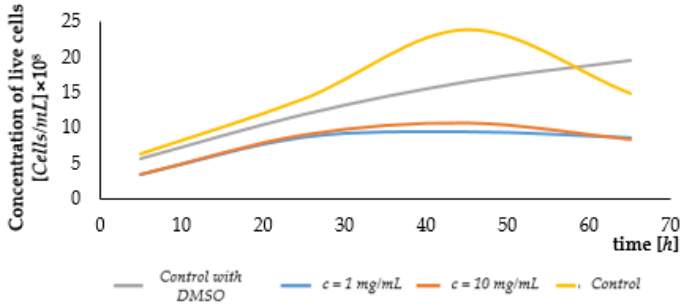

Figure 4: Concentration of live *S. cerevisiae* cells in presence of 6Sac from counting under microscope.

Under microscope we also saw that both 6Sac concentrations (1 mg/mL and 10 mg/mL) have impacted the *S. cerevisiae* viability (percentage of live cells out of all cells under microscope) (**Table 1**). The concentration of 1 mg/mL lowered the yeast cells viability in the first 5 h, then this was stable for some time. The greatest difference between viability in presence of 6Sac and viability of control (broth with DMSO) was observed at 65 h. On the other hand, the concentration of 10 mg/mL had the greatest impact in the first 5 h (**Table 1**). Later we can see higher viability, but at the end, at 65 hours, there is lower viability again, which is also very comparable with the viability of a 1 mg/mL (**Table 1**). The *p*-value also confirms what has been described. For most measurements (exception is viability with 1 mg/mL 6Sac at 5 hours), the *p*-values are less than 0.05, indicating that there is a greater than 95% chance that the difference in viability between culture treated with 6Sac and the control is statistically significant (**Table 1**).

Table 1: *S. cerevisiae* viability.

	Viability (%) ± Standard deviation (*p*-value)		
Time (h)	*Control*	*1 mg/mL [6Sac]*	*10 mg/mL [6Sac]*
5	95.74 ± 4.52	88.71 ± 9.54 (p = 0.266006)	80.30 ± 11.54 (p = 0.005470)
25	98.96 ± 1.79	86.71 ± 7.89 (p = 0.002355)	85.54 ± 14.61 (p = 0.016322)
45	97.78 ± 2.98	87.13 ± 3.42 (p = 0.001176)	89.89 ± 5.32 (p = 0.011357)
65	95.71 ± 4.06	80.95 ± 4.93 (p = 0.001942)	83.02 ± 6.39 (p = 0.002919)

5.4. Bioactivity of substance 6Sac versus sodium saccharinate (NaSac)

Table 2 compares our results (substance 6Sac) with the results (substance NaSac) from study by Moore and co-workers (1979) which reports on measurements after incubation at 30 °C after 60–65 h. Also, we obtained results after 65 hours, however, we used different concentrations of 6Sac (**Table 2**). We can predict that viability of yeast cells treated with 6Sac at a concentration of 1 mg/mL is comparable to those treated with sodium saccharine at the same concentration, because they observed viability at concentration of 0 and 2 mg/mL and they got results right around our result. However, we cannot say the same for a higher concentration of 6Sac (10 mg/mL). We can see that we got the same effect on viability at both concentration of 6Sac, but they found, that the viability depends on the concentration of NaSac.

Table 2: Bioactivity of 6Sac versus sodium saccharinate (NaSac).

Compound	Concentration of added compound [*mg/mL*]	Viability (%) ± standard deviation
NaSac (Moore et al., 1979)	0	100 ± 9
6Sac (Our study)	1	80.95 ± 4.93
NaSac (Moore et al., 1979)	2	74 ± 8
6Sac (Our study)	10	83.02 ± 6.39
NaSac (Moore et al., 1979)	20	61 ± 8
NaSac (Moore et al., 1979)	100	24 ± 10

6. Conclusion

The effect of Methyl 4-hydroxy-1,1-dioxo-2*H*-1,2-benzothiazine-3-carboxylate (6Sac) on the model organism *S. cerevisiae* was tested by two different methods. The first was to monitor the viability of *S. cerevisiae* cells with methylene blue, which enabled to differ between live and dead cells. We counted cells with the Bürker-Türk counting chamber. And the second was to monitor cultiviability of yeast cells. We counted the number of colonies grown on solid media.

We showed that the compound 6Sac had an effect on *S. cerevisiae* by repressing their growth and increasing the percentage of unviable cells. With both tests, we confirmed a smaller amount of viable cells. In a concentration of 10 mg/mL, 6Sac showed the effect. After 65 h the results at both concentrations were the same. The research also showed that the viability of yeast treated with 1 mg/mL 6Sac is comparable to the one treated with sodium saccharinate, but we cannot say that for higher concentrations (**Table 2**).

Our results indicate that 6Sac is biologically active. Further research is needed to confirm the precise action of this compound. Preliminary tests are of importance due to the rising number of new active substances appearing on the market. We need to find out as much information about them as possible, to see whether they have an effect on human health. Any information from research work is a valuable resource for further studies, on which we build new knowledge.

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Conflicts of Interest: The authors declare no conflict of interest.

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