Synthesis and Bacterial Metabolism of *cis*- and *trans*-2-Alkyl Analogues of Sodium Cyclamate

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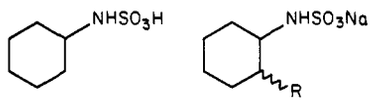
Sodium cyclamate is an effective artificial sweetner, which has been banned from the U.S. market because of alleged carcinogenic properties. It appears that cyclohexylamine, liberated from cyclamate as a result of bacterial metabolism, is the proximate carcinogen. In an effort to elucidate the extent to which analogues of cyclamate would enter into the bacterial metabolic pathway, as well as any stereochemical requirements which might exist, several 2-alkyl analogues of sodium cyclamate were prepared. It was found that *trans*-*N*-(2-methylcyclohexyl)sulfamate (*trans*-**2a**) and *trans*-*N*-(2-ethylcyclohexyl)sulfamate were hydrolyzed by freshly collected fecal suspensions from rats fed cyclamate, but not from control rats, at the same rate as cyclamate itself. *trans*-*N*-(2-Isopropylcyclohexyl)sulfamate (*trans*-**2c**) was not hydrolyzed at all. Surprisingly, two of the analogous cis compounds (*cis*-**2a** and *cis*-**2c**, respectively) *were* hydrolyzed by fecal suspensions from control, as well as from cyclamate-fed, rats. Moreover, *cis*-**2a** was hydrolyzed by incubating it in medium only. Thus, it is apparent that stereochemical influences on the chemical properties of these compounds are substantial. These results do not appear to point the way toward a safe, nonmetabolizable sweetening agent.

Various salts of cyclamic acid (**1**) were used for several



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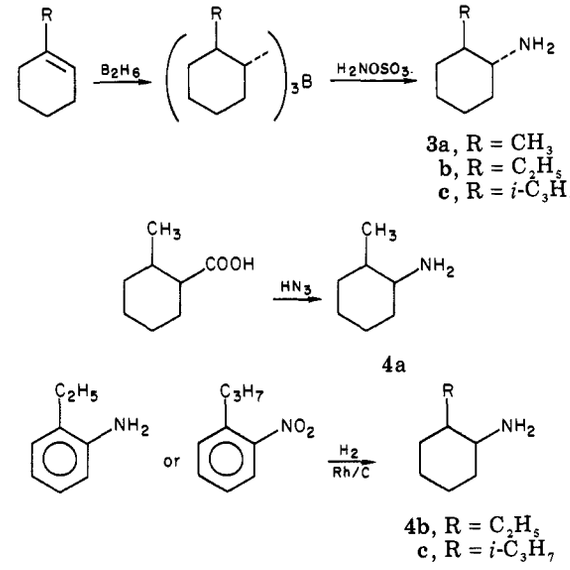
2a, R = CH_3
b, R = C_2H_5
c, R = *i*-C_3H_7

years as artificial sweeteners until a report[1] that bladder tumors were found in 8 out of 80 rats receiving large doses of a mixture of cyclamate and saccharin led to a complete ban on the use of cyclamate in the United States. There is good evidence[2] that the cyclamate metabolite cyclohexylamine is the putative carcinogen; cyclamate is converted in man to cyclohexylamine by a process whose efficiency ranges from minimal to 60%.[3] It is now clear that most of the conversion of cyclamate to cyclohexylamine is accomplished by microorganisms present in the mammalian gastrointestinal tract.[4] The actual mechanism is unknown, but Roxon[5] found that the sulfur from ^{35}S-labeled cyclamate was incorporated into bacterial methionine and suggested that microorganisms use cyclamate as a source of sulfur. It has been shown in the rat that a long period of cyclamate feeding is required in order to maximize the efficiency of conversion of oral cyclamate into urinary cyclohexylamine, and not all rats develop into "converters", regardless of how long cyclamate is fed.[6] This apparently results from the fact that appropriate microorganisms do not grow in all animals. Fresh fecal suspensions from "converter" rats are capable of converting cyclamate to cyclohexylamine, but this ability is rapidly lost if the organisms are grown in culture using standard microbiological techniques.[7] The organism is evidently a mandatory anaerobe, which prefers sulfur sources other than cyclamate. All nutrient media provide these sources of sulfur, so even if rigorously anaerobic conditions are maintained, cyclamate is not hydrolyzed by the microbes.

Although many sulfamates similar to cyclamate are not sweet,[8,9] it has been shown[10] that alkyl substitution on the ring of cycloalkylsulfamates produces compounds retaining varying degrees of sweetness. All the compounds were studied as cis–trans mixtures. Since the organism responsible for production of cyclohexylamine from cyclamate seemed to be rather fastidious, it appeared possible that a sweet compound might be found that would be resistant to microbial hydrolysis. Such a compound would appear to be a safe general-purpose sweetener. Alternatively, if the microbial process were better understood, means might be found to inhibit it. It was the goal of this work to synthesize several *cis*- and *trans*-2-alkylcyclohexylsulfamates and to determine the ability of each to enter into the microbial process that converts cyclamate to cyclohexylamine.

Scheme I



3a, R = CH_3
b, R = C_2H_5
c, R = *i*-C_3H_7

4a

4b, R = C_2H_5
c, R = *i*-C_3H_7

Chemistry. The desired sulfamates **2** were obtained by treating the corresponding amines with chlorosulfonic acid.

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Table I. Conversion of *N*-Cycloalkylsulfamates to Cycloalkylamines by Rat Fecal Suspensions

compd	R	amine formed, μg ± SE (*n*), in 10 mL-incubation: cyclamate-fed rats	amine formed, μg ± SE (*n*), in 10 mL-incubation: control rats
sodium cyclamate	H	39.0 ± 2.9 (4)	0 (2)
trans-2a	CH_3	42.0 ± 8.1 (4)	0 (2)
trans-2b	C_2H_5	57.0 ± 23 (4)	0 (2)
trans-2c	i-C_3H_7	0 (2)	0 (2)
cis-2a	CH_3	457 ± 30 (10)	402 ± 2.0 (2)
cis-2b	C_2H_7	35.0 ± 1.7 (4)	*a*
cis-2c	i-C_3H_7	37.5 ± 2.6 (4)	27.9 ± 0.55 (2)

[a] Not measured.

The way this reaction was reported to have been done[8] required 3 equiv of amine; one constitutes the product, and the others neutralize byproduct acids formed in the reaction. Since our amines were not easily obtained, we wished to make the procedure more efficient. This was accomplished by equilibrating the relatively easily handled cyclohexylamine hydrochlorides with a large excess of triethylamine, a stronger base. On addition of chlorosulfonic acid, only the primary amine was capable of forming the desired sulfamate; the excess triethylamine maintained the necessary neutral reaction milieu. In this way, good yields of sulfamates were obtained by using equimolar amounts of cyclohexylamine and chlorosulfonic acid.

The required *trans* amines **3** were available from the corresponding 1-alkylcyclohexenes by the procedure of Brown,[11] who showed this method to be stereospecific (Scheme I). *cis*-2-Methylcyclohexylamine (**4a**) was prepared according to the procedure of Dauben.[12] Although the properties of our material agreed exactly with those reported, we desired to determine unequivocally the extent to which these cis amines were contaminated with trans material. Therefore, we devised a gas chromatographic procedure capable of discriminating between the acetamide derivatives of the cis and trans amines. This procedure showed all the cis amines to be at least 95% cis material.

Biology. Previous assays of cyclohexylamine produced from cyclamate by rat fecal suspensions were carried out by gas chromatography and operated at the limits of detectability. We desired a more sensitive method that would be easily applicable to a variety of amines and, thus, developed the HPLC method described under Experimental Section. In this assay, the key element is reaction of the amine with phenyl isocyanate to intensify its ultraviolet chromophore, after which the resulting *N*-phenylcycloalkylurea was quantitated using an HPLC external standard method.

Using this method, we measured the extent of conversion of the analogues to the various cycloalkylamines using fresh fecal suspensions from rats fed 1% sodium cyclamate in their drinking water; the results are shown in Table I. It is seen that the apparent extent of conversion of all compounds, except *trans*-**2c**, is at least as great as that of sodium cyclamate itself. We decided to ensure that these apparent rates of conversion were, in fact, the result of enzymatic conversions due to cyclamate-induced bacteria and, therefore, carried out the control experiments shown in the table. To our surprise, both cis compounds tested were hydrolyzed as rapidly in the presence of suspensions from control as from cyclamate-treated rats. No hydrolysis of cyclamate or any of the trans analogues was observed. Unfortunately, insufficient stocks of *cis*-**2b** were available for testing. These data raise the possibility that the hydrolysis of the cis compounds is nonenzymatic. Preliminary experiments reveal significant conversion of *cis*-**2a** and *cis*-**2c**, but not sodium cyclamate, to the corresponding amines when incubated in the presence of medium only for 24 h (agitated by bubbling CO_2). Thus, pronounced and unexpected chemical differences between these cis and trans analogues are apparent; however, with the possible exception of *trans*-**2c**, which requires further study, it does not appear that this series of compounds will present a sweet, nonmetabolizable cyclamate analogue.

Efforts were made to culture the organism involved in this transformation. This is a formidable problem. The organism is apparently a mandatory anaerobe and prefers almost any other sulfur source to cyclamate, yet one can only identify it if cyclamate hydrolysis can be observed. A gram-positive rod capable of hydrolyzing cyclamate was observed in 12–18-h cultures but could not be maintained longer nor identified conclusively.

Experimental Section

Chemistry. Infrared spectra were taken on Beckman IR-33 and Perkin-Elmer Model 727 spectrometers; NMR spectra were recorded on Varian EM-360 and T-60 spectrometers. Mass spectra were obtained on a Varian MAT CH- 5B spectrometer, and gas chromatography data were obtained on a Varian Model 3700 flame-ionization instrument. Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. Microanalyses were performed on a Hewlett-Packard Model 185B CHN microanalyzer at the University of Kansas and by the Australian Microanalytical Service, Melbourne. Where analyses are shown by symbols of the elements, calculated and observed results agreed to within ±0.4%.

Hydroxylamine-*O*-sulfonic Acid. In our experience, commercially available material is not sufficiently pure to carry out the aminations described herein. We therefore prepared and standardized this substance according to the procedure of Audrieth.[13] Purities of 85–95% were routinely obtained.

***trans*-2-Alkylcyclohexylamines (3).** These substances were obtained by the method of Brown,[11] with the following modification. Diglyme is used as the reaction solvent, and we found the amine product always to be contaminated with diglyme. To isolate the product, the amine–diglyme mixture was taken up in a small volume of ether, and dry HCl gas was bubbled through the solution; the amine was isolated in good yield as the hydrochloride. The methylamine was identified by preparation of the corresponding benzamide, mp 150.4–151.4 °C (lit.[14] mp 151 °C). The other amines were identified by their spectral characteristics.

3a (R = CH_3): IR (free amine) 3380, 3300, 2920, 2860, 1600, 1440, 1360 cm^{-1}; NMR ($CDCl_3$)/Me_4Si) δ 0.9 (d, 3 H, J = 4 Hz), 1.2 (s, 2 H), 1.2–2.2 (m, 10 H).

3b (R = C_2H_5): IR (free amine) 3370, 3300, 2920, 2860, 1600, 1440 cm^{-1}; NMR (C_6D_6/Me_4Si) δ 0.7 (s, 2 H), 0.9 (t, 3 H, J = 6 Hz), 1.0–2.3 (m, 12 H).

3c (R = i-C_3H_7): IR (free amine) 3400, 3310, 2950, 2880, 1600, 1445, 1385, 1365; NMR (CCl_4/Me_4Si) δ 0.9 (d, 6 H, J = 5 Hz), 1.0 (s, 2 H), 1.0–2.2 (m, 11 H).

***cis*-2-Methylcyclohexylamine (4a).** This was prepared in 66% yield according to a procedure of Dauben,[12] using *cis*-2-methylcyclopropanecarboxylic acid prepared according to Macbeth.[15] The compound was identified as the corresponding benzamide, mp 111–112 °C (lit.[15] mp 110.5–111.5 °C). To assess

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isomeric purity, we subjected the corresponding acetamide to gas chromatography [15% Carbowax 20 M on Chromosorb W (110–120), 6 ft × 0.25 in. glass column, 150 °C, 34 mL of He/min]. Peak area analysis indicated the amine to be 95% cis, 5% trans.

***cis*-2-Ethylcyclohexylamine (4b).** This substance was prepared by reduction of *o*-ethylaniline by the rhodium-catalyzed hydrogenation procedure of Freidlin,[16] the isolation procedure was modified as follows. To the crude product was added 10 mL of 40% aqueous NaOH. The amine was extracted with ether, the ether solution was dried (K_2CO_3), and the ether was evaporated. Gas chromatographic analysis of the acetamide derivative (conditions as in **4a**) indicated the product to be 87% cis. The isomeric purity was improved by preparing the benzenesulfonamide derivative, using the procedure of King.[17] Three recrystallizations of the sulfonamide, followed by hydrolysis, afforded the amine product, which was 99.2% cis: IR 3380, 3300, 2920, 2860, 1600, 1440; NMR (C_6D_6/Me_4Si) δ 0.6 (s, 2 H), 0.8 (t, 3 H, J = 5 Hz), 1.0–2.0 (m, 11 H), 2.85 (s, 1 H).

***cis*-2-Isopropylcyclohexylamine (4c).** This amine was obtained from *o*-nitrocumene[18] by the same reduction conditions as for **4b**. Gas chromatography (same conditions as in **4a**, except a temperature of 185 °C and a flow rate of 50 mL/min) indicated the product to be 95.3% cis: IR 3400, 3320, 2940, 2880, 1620, 1445, 1385, 1370; NMR (CCl_4/Me_4Si) δ 0.9 (d, 6 H, J = 5 Hz), 1.1 (s, 2 H), 1.0–2.0 (m, 10 H), 3.2 (s, 1 H).

Sodium *N*-(2-Alkylcyclohexyl)sulfamates (2). The general procedure for preparation of these compounds was as follows. To 0.01 mol of amine hydrochloride was added 0.1 mol of triethylamine, and the mixture was stirred for 15 min at room temperature. Chloroform (20 mL) was added, the mixture was cooled to −5 °C, and freshly distilled chlorosulfonic acid (0.01 mol) was added dropwise so as to maintain the temperature below 0 °C. The chloroform was evaporated, and the mixture was treated with 0.03 mol of NaOH in 16 mL of water. The solution was evaporated to dryness, and the solid residue was extracted with 50 mL of boiling absolute ethanol. Following evaporation of the solvent, the sodium sulfamates were obtained in 55–70% yield. Analytical data on these compounds were obtained as follows. *trans*-**2a**: Anal. ($C_7H_{14}NO_3SNa$) C, H, N. *cis*-**2a**: Anal. ($C_7H_{14}NO_3SNa{\cdot}H_2O$) H, N; C: calcd, 36.04; found, 36.49. *trans*-**2b**: Anal. ($C_8H_{17}NO_3S$) C, H, N. (*trans*- and *cis*-**2b** were converted to the free acid form for analysis by using Dowex-50, followed by recrystallization from ethyl acetate/hexane.) *cis*-**2b**: Anal. ($C_8H_{17}NO_3S$) C, H, N. *trans*-**2c** Anal. ($C_9H_{18}NO_3S$ Na) C, H, N. *cis*-**2c**: Anal. ($C_9H_{18}NO_3SNa$) C, H, N.

Biology. Rats were allowed free access to lab chow and to water containing 1% sodium cyclamate and were housed in groups in solid-bottom cages to encourage coprophagy. Animals were kept at least 6 weeks before the ability of fecal suspensions from them to metabolize cyclamate was measured, and nonconverters were gavaged three times with fecal suspensions from converter rats before being discarded. Only converter rats were used in analogue assay.

Freshly collected feces (about 5 g) from converter rats were added to 10 mL of prereduced, anaerobically sterilized, minimal medium (pH 7 ± 0.2) containing resazurin as an oxygen indicator[19] in a sterile 50-mL centrifuge tube; the tube was weighed to determine feces weight. A further 10 mL of medium was added, and the fecal pellets were broken up. The mixture was centrifuged at 500 rpm for 10 min, and the supernatant was decanted into a sterile centrifuge tube. The mixture was centrifuged at 11 000 rpm for 20 min. The pellet was resuspended in a volume of medium equal to the weight of the feces collected. Incubation tubes containing 9 mL of minimum medium and 100 mg of the cyclamate analogue to be tested were incubated with 1.0 mL of the bacterial suspension for 22–24 h at 44–45 °C; the contents of the tubes were agitated by slow streams of CO_2 (1 bubble/s) being bubbled through them.

The entire contents of the tubes were transferred to a screw-capped centrifuge tube and centrifuged 20 min at 2400 rpm. Five milliliters of the supernatant was transferred to another centrifuge tube, and the pH was adjusted to 10 with 3–5 drops of 15% NaOH. Methylene chloride (5 mL) was added, and the tube contents were mixed on a vortex mixer for 3 min. The layers were separated by centrifugation (2400 rpm, 20 min), and the methylene chloride layer was transferred to a clean centrifuge tube. A solution of 0.11 mL of phenyl isocyanate in 50 mL of methylene chloride (0.5 mL) was then added, mixed, and allowed to stand for 1 min, and excess phenyl isocyanate was decomposed by addition of 3 drops of triethanolamine and mixing on a vortex mixer. Phosphate buffer, 0.05 M, pH 10 (5 mL), was then added, the tube contents were mixed, and the tube was centrifuged at 2400 rpm for 20 min. The aqueous layer was aspirated and discarded. The methylene chloride layer was dried (Na_2SO_4), and the methylene chloride evaporated under a stream of nitrogen. Methylene chloride (1.0 mL) and 1.0 mL of a solution of the external standard in methylene chloride (0.02 g of *p*-methoxyacetophenone in 100 mL) were added, and the solution was mixed.

Twenty microliters of this solution was injected into a liquid chromagraph operated under the following conditions: column, 25 × 0.46 cm, silica; detector, UV operated at 240 nm; mobile phase, heptane/2-propanol (95:5); flow rate, 3.2 mL/min. The total amount of amine produced in 10-mL incubations was calculated from the ratio of urea peak height to external standard peak height. In all cases, the standard curve was prepared from solutions of amines carried through the analysis procedure.

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Registry No. **1**, 100-88-9; *trans*-**2a**, 85507-61-5; *cis*-**2a**, 85507-67-1; *trans*-**2a** (Na salt), 56120-30-0; *cis*-**2a** (Na salt), 56120-29-7; *trans*-**2b**, 85507-62-6; *cis*-**2b**, 85507-64-8; *trans*-**2c**, 85507-63-7; *cis*-**2c**, 85507-68-2; *trans*-**2c** (Na salt), 85507-65-9; *cis*-**2c** (Na salt), 85507-66-0; **3a**, 931-10-2; **3b**, 2164-24-1; **3c**, 14898-29-4; **4a**, 2164-19-4; **4b**, 24216-90-8; **4c**, 24216-94-2; 1-methylcyclohexene, 591-49-1; 1-ethylcyclohexene, 1453-24-3; 1-isopropylcyclohexene, 4292-04-0; chlorosulfonic acid, 7790-94-5; *o*-ethylaniline, 578-54-1; *o*-isopropylaniline, 643-28-7.

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