EFFECT OF SOME BENZYL THIOCYANATE ANALOGS ON TETRACYCLINE PRODUCTION

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The importance of benzyl thiocyanate (BTC), $C_6H_5CH_2SCN$, as specific stimulator of the biosynthesis of chlortetracycline by *Streptomyces aureofaciens* was first recognized by PECÁK *et al.*[1,2] in 1958. Addition of this compound to the culture medium at 1~3 μg/ml stimulated the antibiotic production by more than 50% even with high producing strains. Earlier studies showed that glucose oxidation through the pentose cycle becomes a prominent process[2,3] and a decrease in the activity of the enzymes of the tricarboxylic acid cycle occurs in presence of BTC[4]. Subsequently, it was found that BTC increases the specific activity of anhydrotetracycline oxygenase, an enzyme catalyzing a reaction in the biosynthetic pathway to tetracycline[5]. BTC and about forty analogs were examined for their influence on tetracycline formation by *Streptomyces mediolanum* and the results (Table 1) are discussed in this note.

The thiocyanic acid esters described in this work were prepared from the corresponding alkyl chlorides or bromides by treatment with KSCN in boiling EtOH. Productivity evaluations were performed at the flask scale with high producing strains of *S. mediolanum* in a culture medium containing corn meal, starch, corn steep liquor, sunflower oil and mineral salts. BTC was added *via* sunflower oil (0.2 ml of a 1-mg/ml solution) to the previously sterilized culture medium (60 ml) prior to inoculation. The BTC analogs were tested in triplicate at equimolecular amounts (2×10^{-5} M) under the same condition. Flasks were shaken on a rotary shaker (300 rpm) at 28°C for 6 days.

BTC caused a marked stimulatory effect on tetracycline biosynthesis, the peak antibiotic

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Table 1. Improvement of tetracycline formation (%) in presence of 2×10^{-5} M concentrations of the tested substances (*Δ*TS) and BTC as control (*Δ*BTC)*.

Tested substance	*Δ*TS	*Δ*BTC
2-(2-Thienyl)ethyl thiocyanate	24	26
1-Naphthylmethyl thiocyanate	10	30
2-Naphthylmethyl thiocyanate	8	30
2-Benzimidazolylmethyl thiocyanate	9	30
p-Methylbenzyl thiocyanate	8	26
p-Ethylbenzyl thiocyanate	17	44
o-Xylylene dithiocyanate	15	39
m-Xylylene dithiocyanate	2	39
p-Xylylene dithiocyanate	0	39
p-Phenylbenzyl thiocyanate	1	30
p-Fluorobenzyl thiocyanate	1	17
2,3,4,5,6-Pentafluorobenzyl thiocyanate	5	30
p-Bromobenzyl thiocyanate	6	30
p-Methoxybenzyl thiocyanate	3	26
4-Ethoxy-3-methoxybenzyl thiocyanate	0	20
p-Benzyloxybenzyl thiocyanate	0	30
p-Cyanobenzyl thiocyanate	10	26
o-Nitrobenzyl thiocyanate	0	41
m-Nitrobenzyl thiocyanate	5	41
p-Nitrobenzyl thiocyanate	0	41
Methyl thiocyanate	1	20
n-Butyl thiocyanate	0	20
n-Dodecyl thiocyanate	0	20
n-Hexadecyl thiocyanate	0	20
2-Phenylethyl thiocyanate	48	35
3-Phenylpropyl thiocyanate	21	30
2-Methyl-2-phenylethyl thiocyanate	34	30
Cinnamyl thiocyanate	9	29
Phenacyl thiocyanate	19	31
p-Bromophenacyl thiocyanate	10	35
N-(2-Thiocyanoethyl)phthalimide	0	30
Benzyl selenocyanate	26	26
2-Phenylethyl isothiocyanate	11	44
4-Phenylbutyronitrile	6	44
1-Naphthaleneacetamide	5	37
Benzyl mercaptan	0	41

* BTC=Benzyl thiocyanate; TS=tested substance. Stimulatory effect measured by chemical potencies of the culture media at harvest. A=Average chemical potency of the culture medium; B=average chemical potency of the culture medium supplemented with BTC; C=average chemical potency of the culture medium supplemented with the tested compound; $\Delta BTC=(B-A)/A\times100$; $\Delta TS=(C-A)/A\times100$. Flasks of the tested substance were run in parallel against control flasks with and without BTC in each experiment. *Δ*TS and its corresponding *Δ*BTC values are average of 2~4 experiments.

production being reached at a concentration of about 3 μg/ml. At the end of the fermentation period, titers of 8,000~9,000 and 6,000~7,000 μg/ml of tetracycline were usually observed for the test flasks with and without BTC, respectively.

The structures of the tested analogs involve modifications at the benzene nucleus, the polar thiocyano moiety and the aliphatic chain binding them of the prototype BTC molecule. The benzene ring can be replaced by thiophene since 2-(2-thienyl)ethyl thiocyanate was similar in its activity to BTC. However, attempts to modify the aroyl group in other ways invariably led to much less active compounds. Thus, 1- and 2-naphthylmethyl and 2-benzimidazolylmethyl thiocyanates showed less than one third the potency of BTC; and upon introduction of electron-attracting as well as electron-donating substituents into the phenyl group of BTC, the stimulatory effect was largely reduced or even absent. The substituents modify the electronic, steric and lipophilic properties of the parent BTC molecule[6,7], but the way in which these parameters affect the biochemical response is not clear. On the other hand, the aliphatic methyl, *n*-butyl, *n*-dodecyl and *n*-hexadecyl thiocyanates produced essentially no stimulatory effect. It follows from the above results that the hydrophobic benzene nucleus of BTC highly contributes to the activity of the molecule.

Although little information is available concerning the effect of polar groups other than the thiocyano, such a group seems to be highly specific for the BTC action. In 4-phenylbutyronitrile, $C_6H_5(CH_2)_3CN$, where the distance between the phenyl and the polar cyano group is not much different from that in the strong tetracycline stimulators 2-phenylethyl and 3-phenylpropyl thiocyanates, the BTC activity was severely deteriorated. It may be assumed that the low activity exhibited by 4-phenylbutyronitrile is due to the fact that the cyano group *per se* fails to give a firm binding to the receptor. The isomeric 2-phenylethyl isothiocyanate compound, $C_6H_5CH_2CH_2NCS$, in which the sequence of atoms of the polar group has been reversed, also showed a weak stimulatory effect, whereas benzyl mercaptan, $C_6H_5CH_2SH$, was completely devoid of activity. However, the S atom can be replaced by Se without loss of activity. In fact, benzyl selenocyanate, $C_6H_5CH_2SeCN$, prepared by interaction of KSeCN with benzyl chloride, performed at equal-productivity with the corresponding sulfur analog. The carboxamide group can substitute to a small extent for the thiocyano group. During a screening of some plant growth regulators for their influence on tetracycline accumulation in shaken cultures, 1-naphthaleneacetamide, $C_{10}H_7CH_2CONH_2$, exhibited a weak stimulatory action. A possible explanation is that such stimulation is due to a residual BTC-like activity of this compound since other growth-promoting substances showed no productivity-stimulating effect. The related compounds 3-indoleacetic acid, 3-indoleacetonitrile, 3-indolebutyronitrile and 2,4-dichlorophenoxyacetic acid, whose structure would suggest them to possess certain BTC action, did not show activity.

In contrast to the rather high structural demand exhibited by the phenyl and thiocyano groups, their spacing is not apparently too critical for retention of the activity. This accounts for the observation that the homolog compounds with the general structure $C_6H_5(CH_2)_nSCN$ (n=1, 2 or 3) as well as 2-methyl-2-phenylethyl thiocyanate, $C_6H_5CH(CH_3)CH_2SCN$, are all strong tetracycline-stimulating agents. Such molecular species are not rigid, allowing for a considerable variation of the intramolecular distance between the active moieties in extended and folded forms, however, it is possible that the receptor site is also flexible and altered in shape through polarization by the BTC agent. The first homolog 2-phenylethyl thiocyanate yielded the maximum response, *i.e.* about 9% superior to that of BTC, but a decline was observed with the following homolog 3-phenylpropyl thiocyanate. On the other hand, 2-methyl-2-phenylethyl thiocyanate, though still better than BTC, was less active than its pattern 2-phenylethyl thiocyanate, indicating that branching at the interconnecting chain would adversely affect the activity of the molecule. The related compounds cinnamyl, $C_6H_5CH=CHCH_2SCN$ (predominantly *trans*), and phenacyl thiocyanate, $C_6H_5COCH_2SCN$, were significantly less active than BTC. The properties of both the interconnecting chain and the sensitive aroyl moiety have been affected in these compounds. These findings suggest that a non-rigid saturated hydrocarbon chain would facilitate the interaction of the active moieties with the receptor.

The present results show that the phenyl and thiocyano fragments of BTC highly contribute to the activity and their alteration may greatly affect the biological response, whereas some variation is allowed at the interconnecting chain. Compounds possessing a hydrophobic moiety (naphthalene, benzimidazole) and an adequately spaced polar group (cyano, isothiocyano, carboxamide) may also induce a BTC-like response, however, attachment of the unsubstituted phenyl (or thienyl) and thiocyano (or selenocyano) groups at the ends of a lipophilic non-rigid structure of type $(CH_2)_n$, optimally two carbons in length, seems to constitute the best arrangement for a precise fit of the molecule to the receptor and, consequently, maximal response.

Although a number of compounds exhibiting BTC activity were found in the course of this work, most of them were poor tetracycline stimulators compared to BTC. Only 2-phenylethyl thiocyanate produced a significantly higher stimulatory effect. It was also evaluated on the pilot fermentor scale. Fermentations with high producing strains were carried out in 30-liter reactor vessels provided with equipment to monitor and control temperature, pH, dissolved oxygen and, to provide a continual supply of nutrients. In the presence of 2-phenylethyl thiocyanate, the formation of tetracycline reached values average 4.8% higher (number of experiments, 9; standard deviation, 3.23) than those in the BTC control runs.

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