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# CHEMISTRY OF NEOCARZINOSTATIN-MEDIATED DEGRADATION OF d(GCATGC). MECHANISM OF SPONTANEOUS THYMINE RELEASE

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Summary: Identification and quantification of the oxidized deoxyribose moiety associated with spontaneous thymine release in neocarzinostatin-mediated degradation of self-complementary hexanucleotide d(GCATGC) have been described. Criegee-type rearrangement of C-5' hydroperoxide intermediate has been proposed.

Neocarzinostatin (NCS) is an antitumor antibiotic consisting of a protein subunit and nonprotein chromophore.[1] NCS chromophore undergoes irreversible reaction with thiols to generate a biradical species which is capable of cleaving DNA upon aerobic incubation.[1,2] Goldberg and co-workers have demonstrated that (i) among a variety of DNA lesions single strand breaks (T>A>>C>G) resulting from selective oxidation at C-5' of deoxyribose to a nucleoside 5'-aldehyde are the most predominant,[1,3] and (ii) the next most frequent lesion is the formation of 3'- and 5'-phosphate termini with a spontaneous free base release (T>A>>C>G) associated with as yet undetermined form of deoxyribose oxidation.[1,4] While sequence specific interaction of NCS chromophore and accompanying strand cleavage have recently been studied with hexanucleotides,[5] the mechanism of spontaneous free base release is not yet well understood. By the use of self-complementary hexanucleotide d(GCATGC), we have identified and quantitated for the first time the oxidized deoxyribose moiety associated with spontaneous free base release.

In a series of experiments employing various thiols including 2-mercaptoethanol and dithiothreitol, we found that 4-hydroxythiophenol (HTP) is among the best NCS activator for the oligonucleotide modification (Table I).[6] A typical reaction mixture containing NCS (250 μM), HTP (3.5 mM) and double stranded d(GCATGC) (42 μM strand) in 50 mM Tris-HCl buffer (pH 7.2) was incubated for 7 h at 0 °C under aerobic conditions. Direct analysis of the mixture by reverse phase HPLC indicated the formation of two major products d(GCA$_p$) and 5'-aldehyde fragment d(T*GC) (**1**)[3] together with minor amounts of free thymine and d($_p$GC).[7] The structure of **1** was suggested by release of d($_p$GC) upon treatment with hot alkali (0.2 M NaOH, 90 °C) and confirmed by quantitative reduction to d(TGC) by $NaBH_4$. HPLC analysis of the reaction indicated that when one strand of the d(GCATGC) duplex is consumed, the oxidation ceases even after prolonged incubation, resulting in formation of a stoichiometric amount of d(GCAp) (Table I). Further, the formation of **1** (16.4 μM) was considerably less than total d(GCA$_p$) produced (19.9 μM), and the difference corresponded to the amount of spontaneously released thymine (3.2 μM), showing the existence of two pathways for the hexamer degradation (Scheme 1).[1]

When the reaction mixture was reduced with excess $NaBH_4$ immediately after incubation at 0 °C and subjected to enzymatic digestion with alkaline phosphatase, a new stable product **2** was isolated together with d($_p$GC) and d(TGC), the latter resulting from the reduction of **1**. Enzymatic digestion of **2** with snake venom phosphodiesterase followed by alkaline phosphatase quantitatively produced dG and dC in a ratio of 1:1. The structure of **2** was further supported by release of d(GC) upon treatment with hot strong alkali (2 M NaOH, 90 °C) and finally established by comparison of its chemical characteristics, UV spectrum and HPLC behaviors in several solvent systems with those of an authentic sample prepared by independent synthesis from 2(S)-1,2,4-butanetriol.[8] The formation of **2** amounted to 56% of the spontaneously released thymine.

**Scheme 1**

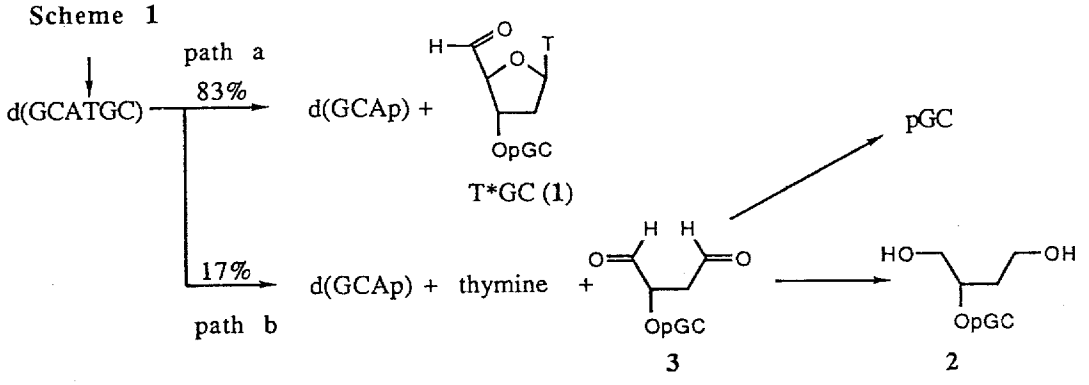


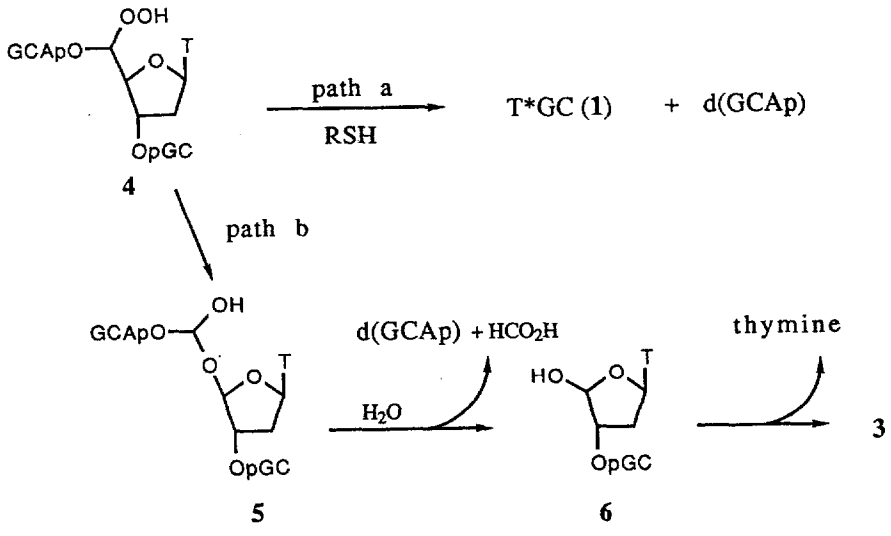

Table I. Quantitative Analysis of Products Formed During NCS-Mediated Degradation of d(GCATGC)[a]

| thiol | free base release (μM)[b] C | G | T | A | d(GCAp)[c] (μM) | 1[d] (μM) | 2[e] (μM) | d(pGC)[e] (μM) | hexamer[a] consumed(μM) |
|---|---|---|---|---|---|---|---|---|---|
| HTP (3.5 mM)[f] | - | - | 3.2 | - | 19.9 | 16.4 | 1.8 | 1.5 | 20.0 |
| ME (20 mM)[g] | - | - | 1.8 | - | 8.9 | 7.2 | 0.6 | 1.1 | 9.1 |
| DTT (5 mM)[h] | - | - | 1.1 | - | 5.7 | 4.8 | 0.3 | 0.7 | 6.0 |

[a] The reaction mixture (50 μl) containing NCS (250 μM), d(GCATGC) (42 μM strand) and a thiol in 50 mM Tris-HCl buffer (pH 7.2) was incubated at 0 °C for 7 h under identical conditions. Analysis of the mixture was effected by reverse phase HPLC (ODS column, 0.05 M triethylammonium acetate containing 3 to 14 % acetonitrile, linear gradient). [b]HPLC condition; ODS column, 0.05 M ammonium formate. [c]Quantitated after alkaline phosphatase digestion. [d]Quantitated after treatment with 40 mM $NaBH_4$. [e]Quantitated after treatment with 40 mM $NaBH_4$ and alkaline phosphatase digestion by HPLC (0.05 M ammonium formate containing 2.5% acetonitrile). [f]4-Hydroxythiophenol. [g]2-Mercaptoethanol. [h]Dithiothreitol.

Isolation and characterization of 2 as well as the fact that spontaneously released thymine is always equal to the sum of d(pGC) plus 2 indicate that the alkaline labile abasic product associated with spontaneous thymine release is dialdehyde 3 which gradually releases d(pGC) during incubation.[9,10] Table 1 also illustrates that the portion of 5'-aldehyde formation is 83% (path a), whereas path b amounts to 17% of the total oxidation. These observations including stoichiometry of the product formation suggest strongly that the observed products must arise from a common precursor.[1,12] Formation of 3 clearly involves C-4'-C-5' cleavage of the deoxyribose moiety. The most reasonable interpretation of the C-4'-C-5' cleavage appears to be a proton-assisted Criegee-type rearrangement[13] of the common C-5' hydroperoxide intermediate 4 to hemiketal 5 which liberates d(GCAp) and 6 spontaneously (Scheme 2).[15] Consistent with this hypothesis, formation of 2 and free thymine release are pH dependent. Incubation with NCS at higher pH, *e.g.*, pH 8.5, retarded the production of 2 and free thymine release as revealed by analysis of the mixture after $NaBH_4$ reduction. The hydroperoxide 4 could result from hydrogen abstraction from C-5' of deoxyribose by NCS-chromophore-derived biradical[2b] followed by dioxygen addition and subsequent hydrogen abstraction by the resulting peroxy radical from thiol. Reductive decomposition of 4 with excess thiol would provide 1.

**Scheme 2**




The present results clearly demonstrated that NCS recognizes AT site of even small hexanucleotide d(GCAT4GC) and thiol-activated NCS induces DNA modification specifically at T4 on one strand. Oxidative DNA damage initiated by C-5' hydrogen abstraction of deoxyribose as described here may have important implications in the action of other structurally related antitumor drugs such as calicheamicin where hydrogen abstraction from C-5' of deoxyribose on one strand has been proposed.[17]

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(6) The order, HTP >> dithiothreitol > 2-mercaptoethanol > thiophenol, for the efficiency of NCS activation was also observed in nicking of sonicated calf thymus DNA and covalently closed circular DNA.
(7) A similar sequence specific strand cleavage has also been reported for certain non-self-complementary hexanucleotides.[5]
(8) **2** was synthesized from 2(S)-1,4-di(4,4'-dimethoxytrityl)1,2,4-butanetriol by conventional phosphotriester method. **2**: Reverse phase HPLC (Cosmosil $5C_{18}$ ODS, 0.05 M triethylammonium acetate containing 2.5% acetonitrile, flow rate 1.5 ml/min, retention time 19.5 min); FABMS, 725 (M+2); $\lambda_{max}$ ($H_2O$) 255 nm ($\varepsilon$ = 17940); 400 MHz $^1H$ NMR ($D_2O$) $\delta$ 1.79 (m, 2 H), 2.28 (m, 1 H), 2.40, (m, 1 H), 2.48 (m, 1 H), 2.85 (m, 1 H), 2.88 (m, 2 H), 3.62 (t, J = 6.7 Hz, 2 H), 4.11 (m, 4 H), 4.16 (m, 1 H), 4.22 (m, 1 H), 4.42 (m, 1 H), 4.58 (m, 1 H), 4.99 (m, 1 H), 5.82 (d, J = 7.5 Hz, 1 H), 6.24 (t, J = 7.1 Hz, 1 H), 6.29 (t, J = 6.8 Hz, 1 H), 7.78 (d, J = 7.5 Hz, 1 H), 8.06 (s, 1 H).
(9) Control experiments indicated that release of d($_p$GC) (7%) during incubation is not due to the spontaneous decomposition of **1** which has been proved to be stable under the incubation conditions, whereas alkali treatment of **1** provided thymine and d(pGC) quantitatively.
(10) Detection of 2-deoxyribonolactone, which is inert to $NaBH_4$, has been reported as ribose oxidation product in NCS-induced cytosine release from d(ACG) sequences of duplex oligonucleotides.[11]
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(13) There is considerable precedent for such proton-assisted Criegee type rearrangement of $\alpha$-alkoxy hydroperoxides as exemplified by the rearrangement of C-4'-hydroperoxynucleosides.[14]
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(15) An alternative mechanism involving $\beta$-cleavage of the alkoxyl radical resulted from homolysis of -O-O- bond of 4 cannot be ruled out.[16]
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