# Notes

## Thienyl and Thiazolyl Acyclic Analogues of 5-Deazatetrahydrofolic Acid

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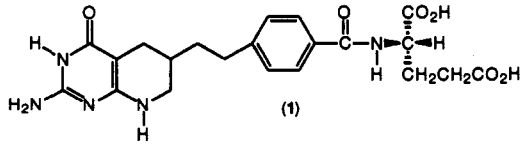
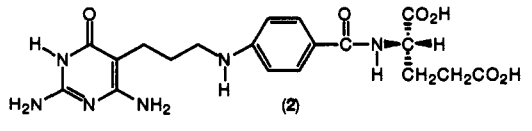
Analogues of *N*-[4-[[3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]benzoyl]-L-glutamic acid (5-DACTHF), in which the phenylene group is replaced by either a thienoyl or a thiazolyl group were synthesized. These compounds were prepared by reductive amination of suitably protected pyrimidinylpropionaldehyde with the aminoaroyl glutamates. These glutamates were in turn synthesized from the corresponding nitroaroyl carboxylic acids by condensation with protected glutamic acid followed by catalytic reduction. The compounds were tested as inhibitors of methotrexate uptake as a measure of binding to the reduced folate transport system, as inhibitors of glycinamide ribonucleotide transformylase, as substrates for folylpolyglutamate synthetase, and as inhibitors of tumor cell growth in cell culture. The thiophene analogue was found to be equal in activity to 5-DACTHF in the MCF-7 cell growth inhibition assay while the thiazole analogue was 9-fold more active. Indeed this thiazole was over 4 times more active in the MCF-7 cell line than the clinically investigated compound 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF).

Recent efforts toward the design of less toxic antifolate chemotherapeutic agents have led to a number of compounds with selective activity against the enzyme glycinamide ribonucleotide transformylase (GAR-Tfase).[1-9] This folate-dependent enzyme catalyzes the first of two one-carbon transfer reactions in the *de novo* purine biosynthetic pathway and represents a novel target for selective cytostatic activity.[22,23] The rationale for expecting selectivity include the observed higher rate of *de novo* purine biosynthesis in cancer cells[10-12] which may indicate an exploitable reliance on this purine source.

Notable among the GAR-Tfase selective compounds is 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF) (1) first reported by Taylor and co-workers.[1] The 6*R* isomer of 1 has recently been advanced to clinical trials as lometrexol. Our group has reported recently[13] the synthesis and biological activity of analogues of *N*-[4-[[3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]benzoyl]-L-glutamic acid (5-DACTHF) (2), which was first prepared by Kelley and co-workers.[8]



(1)



(2)

Data from earlier studies[8,14,15] of 2 and its polyglutamates indicated that activity as an inhibitor of cell growth for 5-DACTHF analogues is a composite function of GAR-Tfase inhibition, the efficiency of transport into the cell by the reduced folate transport system, and the degree of polyglutamation by folylpolyglutamate synthase (FPGS). Increased transport and polyglutamation elevate the intracellular concentration of the compounds, and polyglutamates of 5-DACTHF are more potent inhibitors of GAR-Tfase than the parent compound.[8]

Marsham and co-workers[16] have synthesized quinazoline-based thymidylate synthase inhibitors in which a thiophene or a thiazole group is substituted for the phenyl group in a benzoylglutamate function and reported a 20–45-fold increase in cytotoxicity versus the parent compound despite a 2-fold decrease in enzyme inhibition. This was attributed to increased efficiency of polyglutamation and active transport into the cell by the reduced folate transport system.

Taylor and co-workers[5] reported the synthesis of acyclic analogues of 1, 9, and 10 including compounds utilizing a 2,5-thiophenylene or 2,5-furanyl linking group in place of the phenylene group. None of these compounds proved as active *in vitro* as the parent 1. However, replacement of the phenylene group of 1 with 2,5-furanyl or 2,5-thiophenylene gave compounds 11, and 12 ranging from 2-fold less active to 7-fold more active than the parent.[20]

We report here the synthesis of two 5-DACTHF analogues in which the phenyl ring of the benzoyl glutamate function is replaced with thiophene and thiazole: *N*-[[5-[[3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]-2-thienoyl]carbonyl]-L-glutamic acid (**8a**) and *N*-[[5-[[3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]-2-thiazolyl]carbonyl]-L-glutamic acid (**8b**). These compounds were evaluated as inhibitors of GAR-Tfase and as substrates for FPGS and the reduced folate transport system and tested *in vitro* against MCF-7 breast adenocarcinoma.

### Chemistry

Both target molecules were prepared similarly to the lead compound 5-DACTHF (2) (Scheme 1). 5-Ni-

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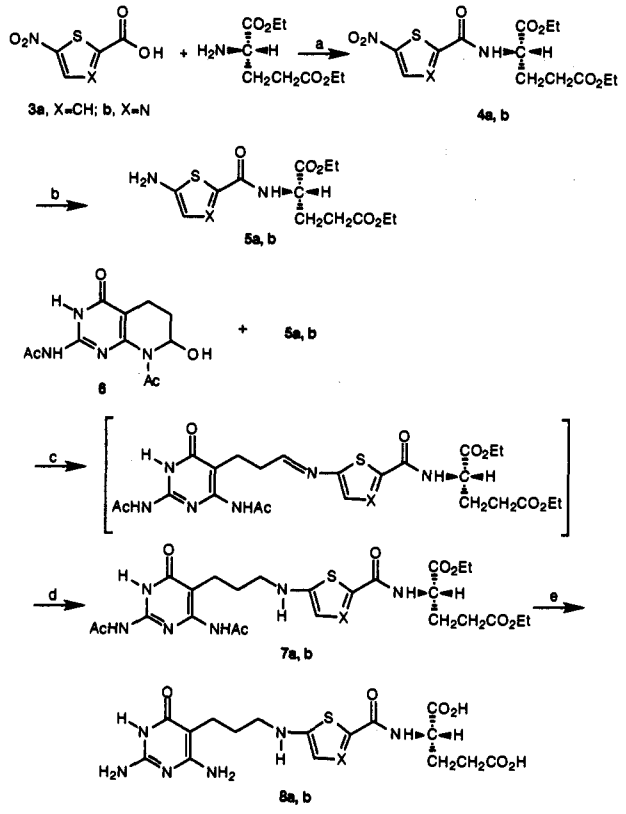
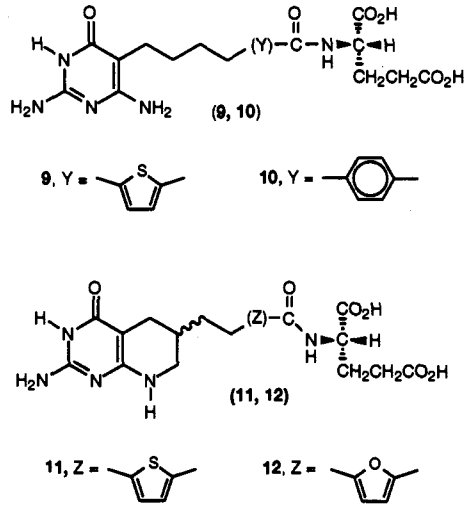
**Scheme I**[a]




[a] Reagents: (a) 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride/triethylamine/$CH_2Cl_2$; (b) 10% Pd–C/EtOH/$H_2$; (c) 3-Å mol. sieve/HOAc/EtOH; (d) $NaCNBH_3$; (e) (i) 1.0 N NaOH/EtOH, (ii) 1.0 N HCl.




trothiophene- and -thiazolecarboxylic acids (**3a, b**) were coupled with diethyl L-glutamate to give amides **4a,b**, which were reduced catalytically to the amino derivatives **5a,b** in good yield.

Reductive amination using these deactivated amines proved difficult; stirring the hemiaminal **6** and **5a** or **5b** with acetic acid in the presence of 3-Å molecular sieves gave the optimum conversion to intermediate imines as monitored by $^1$H-NMR. Reduction with sodium cyanoborohydride proceeded more cleanly when the mixtures were warmed and the reducing agent was added portionwise over an extended time. Silica gel chromatography gave the *N*-acetyl esters **7a,b**; however, HPLC, NMR, and mass balance indicated that substantial material remained on the silica gel column and could not be eluted even with very polar solvents. Loss of one or both protecting acetyl groups is thought to cause this binding, and the result was modest yields (ca. 50%). Hydrolysis of the esters also resulted in partial decomposition and made purification by reverse-phase semipreparative chromatography necessary to obtain a second crop and improve recovery. In the case of **8a**, overall yield for the reductive amination and hydrolysis steps was 42%. For **8b**, a second crop was not pursued and the overall yield for these two steps was only 17%.

Table 1. *In Vitro* Activity

| compd no. | $IC_{50}$, μM: GAR-fase[a] | MTX uptake[b] | cell growth[c] | FPGS:[d] $V_{max}/K_m$, %/μM |
|---|---|---|---|---|
| 1 | 0.22 | 1.5 | 18 | 97/17.5 |
| 2 | 3.00 | 1.2 | 37 | 106/7.3 |
| 8a | 2.06 | 1.6 | 38 | 118.3/9.3 |
| 8b | 0.14 | 0.8 | 4 | 99.3/8.6 |

[a] Hog liver GAR transformylase with (6*R*)-10-formyl-FH4 as cofactor.[8] [b] Inhibition of [$^3$H]methotrexate transport into MOLT-4 cells.[19] [c] Inhibition of growth of MCF-7 human breast adenocarcinoma using continuous exposure for 72 h. [d] Hog folylpolyglutamate synthase; $V_{max}$, %, is relative to aminopterin.[8]

Table II. *In Vitro* Antitumor Activity

| compd no. | cell growth CCRF/CEM $IC_{50}$, nM | ref no. |
|---|---|---|
| 1 | 16 | 5 |
| 1* | 15 (6*R* diastereomer, Lometrexol) | 20 |
| 9 | 59 | 5 |
| 10 | 132 | 5 |
| 11 | 2.3 | 20 |
| 12 | 27 | 20 |
| 2 | 198 | 5 |

## Biological Discussion

Biological data for compounds **1**, **2**, **8a**, and **8b** are summarized in Table 1. Thiophene analogue **8a** and lead compound **2** are nearly identical in inhibitory activity against the MCF-7 cell line and are about 2-fold less active then DDATHF (**1**). Thiazole analogue **8b**, however, is 4.5 times more potent than **1** in this cell line. Comparison of substrate activity for FPGS shows all four of these compounds are nearly equal in activity. However, **1** and **8b** are each at least 10-fold more inhibitory against target enzyme GAR-Tfase than **8a** or **2**. Thiazole **8b** is also 2-fold more active in the active transport assay (MOLT-4) than **1**, and this may also contribute to the measured $IC_{50}$ of 4 nM in the MCF-7 cell line.

The acyclic analogues of DDATHF (**1**), **9** and **10** recently reported by Taylor *et al.*[5] as well as the heteroaryl analogues **11** and **12**,[20,21] were evaluated in different cell lines than were our compounds (**8a,b**), making direct comparison of growth inhibition impossible. Comparison of the data in Tables 1and 2indicates that differences in the cell line used for growth inhibition tests can have a large effect on $IC_{50}$ values (i.e., DACTHF (**2**) is only 2-fold less active than **1** against MCF-7 cell line (Table 1) but 12-fold less active in the CCRF/CEM cell line). Substitution of 2,5-thiophenylene for phenylene in the cyclic **1** and acyclic **10** compounds gave products **11** and **9** having a 2-fold and 7-fold increase in cytostatic activity, respectively. In contrast, this substitution in our acyclic compound **2** gave no improvement in activity. The 2-fold poorer activity of the furanyl derivative **12** versus the parent **1** in contrast to 7-fold increased activity in **11** further

demonstrates the sensitivity to change in this region of these molecules. It should be further noted that 2,4-linked isoteres of 2,5-linked compounds 9 and 11 were less active.[5,21] In conclusion, two heteroaryl derivatives of 5-deazaacylotetrahydrofolic acid (2) were prepared and found to be potent cytostatic agents in the MCF-7 cell line. In general, replacement of phenylene with thiophenylene and thiazole leads to progressively more active compounds. This trend is seen both in the folate like GAR-Tfase inhibitors and in the quinazoline-based TS inhibitors. Further improvements in potency may be realized by substituting other heteroaryl groups for phenylene in 1, 2, or 10.

## Experimental Section

NMR spectra were obtained on Varian XL200 or XL300 spectrometers. Elemental analyses (Atlantic Microlabs, Inc., Atlanta, GA) were within 0.4% of theoretical values unless noted otherwise.

**Diethyl *N*-(5-Nitro-2-thienoyl)-L-glutamate (4a).** 5-Nitrothiophene-2-carboxylic acid[18] (**3a**) (27.1 g, 156.5 mmol), L-glutamic acid diethyl ester hydrochloride (37.5 g, 156.5 mmol), and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (30.0 g, 156.5 mmol) were stirred in dry $CH_2Cl_2$ (350 mL) under nitrogen at 0 °C. Triethylamine (21.8 mL, 156.5 mmol) was added dropwise over 1 h, and the mixture was allowed to warm to room temperature. After 2 h, $CH_2Cl_2$ (1 L) was added, and the solution was washed with 0.05 N HCl (3 × 100 mL), saturated $NaHCO_3$ (3 × 100 mL), and water (3 × 100 mL). The solution was dried with $MgSO_4$, filtered, and evaporated *in vacuo* to give diethyl *N*-(5-nitro-2-thienoyl)-L-glutamate (**4a**) (48.8 g, 88%) as an amber oil. $^1$H-NMR ($Me_2SO$-$d_6$): δ 1.17 (m, 6H, $OCH_2CH_3$), 1.9-2.2 (m, 2H, $CH_2CH_2CO_2Et$), 2.46 (m, 2H, $CH_2CO_2Et$), 4.41 (m, 4H, $OCH_2CH_3$), 4.44 (m, 1H, CH), 7.93 (d, 1H, $J$ = 4 Hz, thiophene $C_3H$), 8.17 (d, 1H, $J$ = 4 Hz, thiophene $C_4H$), 9.26 (d, 1H, $J$ = 7 Hz, glutamic acid NH). Anal. C, H, N, S.

**Diethyl *N*-(5-Amino-2-thienoyl)-L-glutamate (5a).** Diethyl *N*-(5-nitro-2-thienoyl)-L-glutamate (**4a**) (28.0 g, 78.1 mmol) was dissolved in EtOH (600 mL) and treated with 10% palladium on carbon (6.0 g). The mixture was hydrogenated on a Parr apparatus for 18 h at 40 psi. Filtration on Celite followed by evaporation gave diethyl *N*-(5-amino-2-thienoyl)-L-glutamate (**5a**) (22.5 g, 88%) as a purple oil. $^1$H-NMR ($Me_2SO$-$d_6$): δ 1.2 (t, 6H, $J$ = 6 Hz, $OCH_2CH_3$), 1.85–2.15 (m, 2H, $CH_2CH_2CO_2Et$), 2.4 (m, 2H, $CH_2CH_2CO_2Et$), 4.08 (q, 4H, $J$ = 6 Hz, $OCH_2CH_3$), 4.31 (m, 1H, CH), 5.83 (d, 1H, $J$ = 4 Hz, thiophene $C_3H$), 6.3 (br s, 2H, $NH_2$), 7.42 (d, 1H, $J$ = 4 Hz, thiophene $C_4H$), 8.12 (d, 1H, $J$ = 7 Hz, glutamatic NH). Anal. C, H, N, S.

***N*-[5-[[3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]-2-thienoyl]-L-glutamic Acid (8a).** Diacetylhemiaminal[8] (**6**) (17.8 g, 66.9 mmol) and diethyl *N*-(5-amino-2-thienoyl)-L-glutamate (**5a**) (22.0 g, 67 mmol) were stirred in EtOH (1.2 L) and treated with acetic acid (32 mL) and activated 3-Å sieves (100 mL, 75 g mL). After 20 h the mixture was warmed to 50 °C, and $NaCNBH_3$ (5.5 g, 87.5 mmol) was added in several portions over a 25-h period. The mixture was filtered warm through Celite and the filtrate evaporated *in vacuo*. The residue was digested in EtOAc (2 L) and filtered, and the filtrate was evaporated onto 60 g of silica gel. Chromatography on 600 g of silica gel in EtOAc and EtOAc/MeOH (4/1) gave two fractions (15.0 and 8.0 g). The larger fraction was pure by $^1$H-NMR with spectral data that was consistent with structure **7a**. The smaller fraction contained an unknown contaminant (ca. 15%). The larger fraction was dissolved in EtOH (400 mL) treated with 1.0 N NaOH (400 mL) and warmed to 40 °C. After 18 h, the mixture was cooled to 0 °C and filtered, and the filtrate was acidified to pH 3.5 by slow addition of 1.0 N HCl. The suspension was reduced *in vacuo* to 100 mL and filtered, and the filtercake was washed with several small portions of water and dried *in vacuo* to yield *N*-[5-[3-(2,4-diamino-1-6-oxo-5-pyrimidinyl)propyl]amino]-2-thienoyl]-L-glutamic acid (**8a**) (8.62 g, 28%) as a white powder. $^1$H-NMR ($Me_2SO$-$d_6$): δ 1.58 (m, 2H, $CH_2CH_2CH_2CO_2H$), 1.7-2.2 ($CH_2CH_2CO_2H$) 2.22–2.38 (m, 4H, $CH_2CH_2CO_2H$ and $CH_2CH_2CH_2NH$), 4.28 (m, 1H, C*H*), 5.79 (d, 1H, $J$ = 4 Hz, thiophene $C_3H$), 5.8 (br, 2H, $NH_2$), 6.5 (br, 2H, $NH_2$), 6.95 (br, 1H, aminothiophene NH), 7.47 (d, 1H, $J$ = 4 Hz, thiophene $C_4H$), 8.0 (d, 1H, $J$ = 7 Hz, glutamic acid NH), 9.9 (br, 1H, $N_1H$), 12.3 (br, 2H, glutamic acid, $CO_2H$'s). Anal. C, H, N, S.

Similar treatment of the smaller, impure fraction gave crude product that was dissolved in water (20 mL) by addition of a minimum amount of triethylamine and chromatographed on a reverse-phase column (Waters Prep 500 $C_{18}$) using a mobile phase of 10% methanol, 90% water, and 0.1% (v/v) triethylammonium trifluoroacetate. The pure fractions were combined and washed with $CH_2Cl_2$ and the aqueous phase acidified to pH 3.5 with 1.0 N HCl. Filtration gave a white solid that was rinsed with water and dried in vacuo to yield an additional 4.4 g (14%) of product **8a** with satisfactory microanalysis.

**Diethyl *N*-[(5-Nitro-2-thiazolyl)carbonyl]-L-glutamate (4b).** 5-Nitro-2-thiazole carboxylic acid[17] (**3b**) (28.26 g, 162.4 mmol), L-glutamic acid diethyl ester hydrochloride (38.95 g, 162.5 mmol), and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (31.12 g, 162.5 mmol) were stirred in $CH_2Cl_2$ (560 mL) under nitrogen at 0 °C. Triethylamine (22.65 mL, 162.4 mmol) was added dropwise over 1 h, and the mixture was allowed to warm to room temperature. After 2 h, $CH_2Cl_2$ (1 L) was added and the mixture washed with 0.05 N HCl (3 × 100 mL), saturated $NaHCO_3$ (3 × 100 mL), and water (3 × 100 mL). The solution was dried with $MgSO_4$, filtered, and evaporated *in vacuo* to give diethyl *N*-[(5-nitro-2-thiazolyl)carbonyl]-L-glutamate (**4b**) (52.0 g, 89%) as a tan solid. $^1$H-NMR ($Me_2SO$-$d_6$): δ 1.15 (m, 6H, $OCH_2CH_3$), 2.12 (m, 2H, $CH_2CH_2CO_2Et$), 2.28 (t, 2H, $J$ = 7 Hz, $CH_2CH_2CO_2Et$), 4.05 (m, 4H, $OCH_2CH_3$), 4.48 (m, 1H, CH), 9.0 (s, 1H, ArH), 9.6 (d, 1H, $J$ = 7.5 Hz, NH). Anal. C, H, N, S.

**Diethyl *N*-[(5-Amino-2-thiazolyl)carbonyl]-L-glutamate (5b).** Diethyl *N*-[(5-nitro-2-thiazolyl)carbonyl]-L-glutamate (**4b**) (35.8 g, 99.6 mmol) was dissolved in EtOH (500 mL) and treated with 10% palladium on carbon (36.0 g). The mixture was hydrogenated at 50 psi for 3 h. Filtration on Celite followed by evaporation gave diethyl *N*-[(5-amino-2-thiazolyl)carbonyl]-L-glutamate (**5b**) (22.6 g, 67%) as a red oil. $^1$H-NMR ($Me_2SO$-$d_6$): δ 1.12 (m, 6H, $OCH_2CH_3$), 2.1 (m, 2H, $CH_2CH_2CO_2$ Et), 2.31 (t, 2H, $J$ = 7 Hz, $CH_2CH_2CO_2Et$), 4.02 (m, 4H, $OCH_2CH_3$), 4.35 (m, 1H, CH), 6.46 (s, 2H, $NH_2$), 6.85 (s, 1H, ArH), 8.42 (d, 1H, $J$ = 7.5 Hz, NH). Anal. C, H, N, S.

***N*-[[5-[[3-(2,4-Diamino-1,6-dihydro-6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]-2-thiazolyl]carbonyl]-L-glutamic Acid (8b).** Diacetylhemiaminal[8] (**6**) (14.2 g, 53.5 mmol) and diethyl *N*-[[5-amino-2-thiazolyl)carbonyl]-L-glutamate (**5b**) (9.6 g, 29 mmol) were stirred in EtOH (500 mL) and treated with acetic acid (15 mL) and activated 3 Å sieves (300 mL, 225 g mL), and the mixture was warmed to 35 °C. After 18 h the mixture was warmed to 50 °C, and $NaCNBH_3$ (6.0 g, 95.5 mmol) was added in several portions over 30 h. the mixture was filtered warm through Celite, and the filtrate was evaporated *in vacuo*. The crude mixture was digested in EtOAc (1 L) and filtered, and the filtrate was evaporated onto 70 g of silica gel. Chromatography on 500 g of silica gel in EtOAc and EtOAc/MeOH (6/1) gave a fraction (9.9 g) pure by $^1$H-NMR with spectral data consistent with structure **7b**. This fraction was dissolved in 1.0 N NaOH (180 mL) and warmed to 35 °C for 40 h. The solution was cooled to 0 °C, acidified with 1.0 N HCl to pH 3.5, and reduced *in vacuo* to 100 mL volume. The suspension was filtered, and the filtercake was washed with water and dried *in vacuo* to yield *N*-[[5-[[3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]amino]-2-thiazolyl]carbonyl]-L-glutamic acid (**8b**) (2.36 g, 17%) as a pale yellow powder. $^1$H-NMR ($Me_2SO$-$d_6$): δ 1.5–1.7 (m, 2H, $CH_2CH_2CH_2$), 1.85–2.15 (m, $CH_2CH_2CO_2H$), 2.15–2.4 (m, 4H, $CH_2CO_2H$, $CH_2CH_2CH_2N$), 3.05 (m, 2H, $CH_2N$), 4.38 (m, 1H, CH), 5.8–6.0 (br, 2H, $NH_2$), 6.1–6.3 (br, 2H, $NH_2$), 6.87 (s, 1H, thiazole CH), 7.15 (m, 1H, NH), 8.32 (d, 1H, $J$ = 8 Hz, GluNH), 9.5–10.5 (br, 1H, $N_3H$), 11.8–12.8 (br, 2H, $CO_2H$'s). Anal. C, H, N, S.

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