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ALKYLATION OF THE COMPONENTS OF NUCLEIC ACIDS BY ETHYLENEIMINE AND ITS DERIVATIVES. 3.* ALKYLATION OF NUCLEOTIDES

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The products of the alkylation of a number of mononucleotides by ethyleneimine and thiotepa have been separated by ion-exchange chromatography on cellulose and by reversed-phase and ion-exchange HPLCs, and have been identified by acid hydrolysis, UV spectroscopy, and TLC. Under natural conditions, alkylation takes place both at the phosphate group and in the residue of the heterocyclic base of the nucleotide. Acid media lead to an increase in the yield of products modified in the base.

It is generally considered that at pH 6.5-7.0 the phosphate groups of mononucleotides are alkylated by various reagents faster than the heterocyclic nuclei of the bases, since at this pH the phosphate group has a double negative charge and is the stronger nucleophile [2, 3]. However, under the same conditions the alkylation of ATP by ethylene oxide in the terminal phosphate group takes place only half as fast as in the base [4].

As shown in [5, 6], on the use of ethyleneimine and thiotepa as alkylating agents under mild conditions the alkylation of nucleoside phosphates proceeds mainly at the phosphoric acid residue, and, of the bases in DNA and its components, only guanine is modified. With a more prolonged alkylation of DNA (24 h instead of 2), products of modification at adenine residues are also formed [7]. Since until now it has nevertheless remained unclear how, to what degree, and under what conditions bases other than guanine are alkylated as components of nucleotides we undertook the present investigation, in which we have studied the influence of a variation in the conditions (mainly the pH) on the direction of alkylation reactions of mononucleotides.

Curve I of Fig. 1 represents a chromatogram of the products of the alkylation of AMP by thiotepa in the presence of the proton donor $HClO_4$ [8]. Two fractions (A and B) were eluted by water, and the others, including the fraction of unmodified AMP (fraction F) by a concentration gradient of NaCl. On alkylation in the absence of a proton donor, the nature of the chromatogram was retained but the yields of all the fractions apart from fraction F had decreased, while fraction B was totally absent (Fig. 1, curve 3).

The separation of a control mixture containing adenine, adenosine, and AMP under the same conditions showed that water eluted from the column, the base, and the nucleoside (in Fig. 1, fraction A corresponds to this), and then, at an NaCl concentration of 0.08-0.09 M the AMP was eluted. It followed from this that fractions A and B corresponded to hydrolysis products of AMP; as will be shown below, these were mainly alkylated nucleosides. This conclusion was confirmed by the UV spectrum of fraction B, which was completely identical with the spectrum of adenosine alkylated in the base — λ_{max} in an alkaline medium 263, 270, 278, and 300 nm (see [1]).

Since the UV spectra of adenylic acids alkylated at different nucleophilic centers differ little from one another and from the spectrum of unsubstituted AMP [2], to identify the substances in the fractions we subjected them to acid hydrolysis (the

*For Communication 2, see [1].

Abbreviation: thiotepa) triethylenephosphorothioamide.

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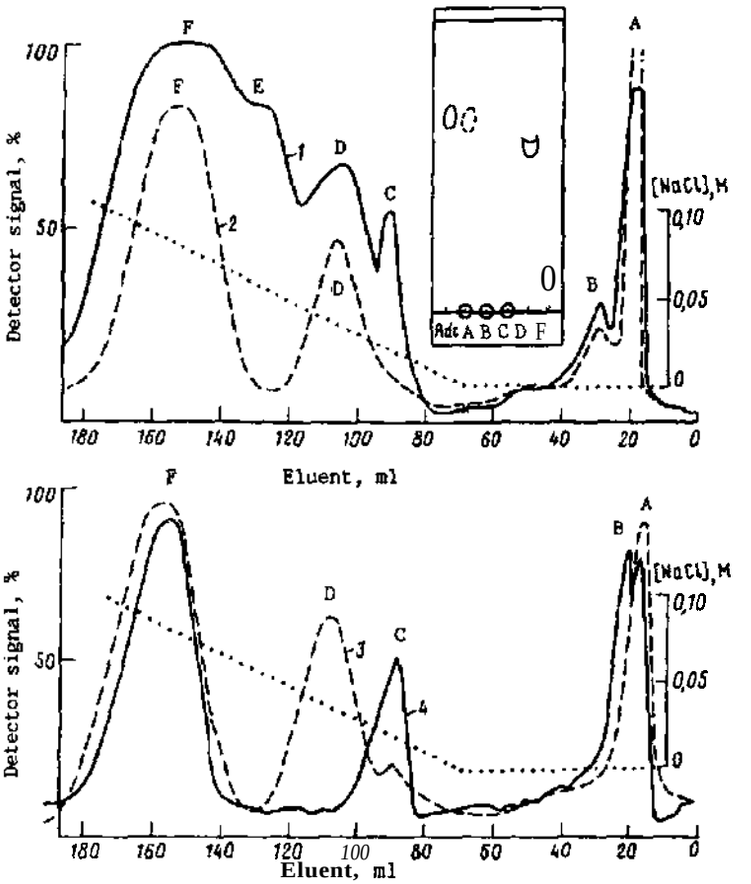
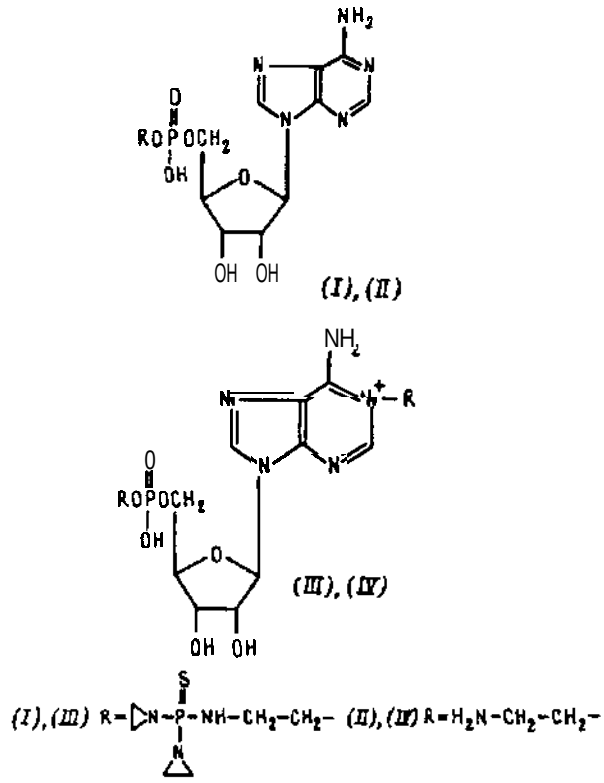


Fig. 1. Chromatography on cellulose DE-52 of the products of the alkylation of AMP in the presence of $HClO_4$ and TLC of fractions A-F of curve 1. Alkylation conditions; ratio of AMP to thiotepa; pH; time (h); and temperature (°C) of the reaction: 1) 1:2; 4.5; 24; 20; 2) 1:2; 4.5; 1 year; 20; 3) 3:2; 8; 5; 37; 4) 1:1; 1; 24; 20. The dotted line shows the NaCl concentration gradient. The symbols A-F are explained in the text.

samples of hydrolysis were taken from the peaks of the curves). The bases formed on hydrolysis were then separated by reversed-phase HPLC. Measurement of the spectra of the bases isolated showed that fractions D in Fig. 1 (the main component of the hydrolysate being adenine) contained AMP alkylated in the phosphate group (I, scheme), while fractions C (the hydrolysis product being the N1-alkyladenine) contained AMP alkylated in the base and in the phosphoric acid residue (III). The latter conclusion was made on the basis of the fact that AMP alkylated only in the base and having, like the unsubstituted nucleotide, two free OH groups in the phosphoric acid residue, should be retained on the column more strongly than the nucleotide substituted in the phosphate residue (fraction C) and be eluted after it (fraction E) (see scheme on top of following page).

The fact is also worth mentioning that after prolonged (~ 1 year) storage of the total alkylated mixture represented by curve 1, peaks C and E had disappeared (curve 2) and, at the same time, the amount of the substance of fraction A had risen through an increase in its content of alkylated adenine.

The ^{13}C NMR spectroscopy of the product from fraction D confirmed that it was AMP alkylated in the phosphoric acid residue; the absence of a shift of the signals of the C2–C3 carbon atoms as compared with the spectrum of the initial AMP, the downfield shift by 1.1 ppm of the C5′ signal of the ribose residue, and the upfield shift by 1.2 ppm of the C4′ signal are characteristic for a transition to diesters [9, 10]. In addition, in the ^{31}P NMR spectrum a pronounced change was observed in the chemical shift of the signal of the phosphorus atom of the nucleotide (of the order of 3 ppm), which is also characteristic tor an alkylated phosphate group. In analogy with the results of other work [5, 6], in the ^{1}H NMR spectrum of the product from fraction D the signals of two methylene groups linked with one another and with a phosphate group, at 6 3.01 and 3.83 ppm,



and a multiplet of the signals of the protons of two ethyleneimine groups, at 6 1.87 ppm, were observed, which also indicated the occurrence of phosphoaminoalkylation.

A study of the influence of different pH values and ratios of the reactants on the direction of the alkylation reactions showed that alkylation in the phosphate group of a nucleotide took place mainly in a weakly alkaline medium and with an excess of AMP (Fig. 1, curve 3). The increase in the amount of fraction A then observed was due to the known lability of nucleotide diesters in an alkaline medium. In neutral and weakly acidic media and, in the case of an excess of thiotepa, weakly alkaline media, as well, the alkylation of AMP proceeded at both nucleophilic centers, and chromatograms of such mixtures repeated curve 1 to a greater or smaller degree. Alkylation at pH 1 led to an increase in the yield of fractions A and B (i.e., hydrolysis products), and the unchanged alkylation product was eluted in the form of the single fraction C (curve 4) containing the nucleotide alkylated in the phosphate group and in the base.

The individual fractions of curve 1 were also analyzed by TLC (see Fig. 1). As can be seen from chromatogram, the product from fraction D had a mobility close to that of adenine, the slight lag and the distorted form of the spot being due to the presence of NaCl in the product, as was confirmed by the TLC of a mixture of adenine and NaCl under the same conditions. The nucleotide alkylated at the phosphate group obviously underwent hydrolysis to adenosine in an alkaline solvent system (pH 11), as was shown by the UV spectrum of an eluate (0.1 N HCl) from the spot, which coincided with the spectrum of unsubstituted adenosine.

The same must be said about fraction C. If this was a product of substitution in the base and in the phosphate group, then, in the given system, it should have been decomposed into an alkyladenosine even more readily, while its zero mobility indicated N1-phosphoaminoethylation. Also in favor of modification at the N1 position are the results of alkylation at pH 1, where reaction at the N^6 position and also a N1 → N^6 rearrangement are impossible. On the use of a neutral solvent system (system A, described in [1]) the alkylation products were not separated from AMP.

According to TLC, fraction A was inhomogeneous and contained both unsubstituted adenosine and adenosine alkylated in the base, while fraction B contained only adenosine modified in the heterocycle. Thus, the nucleoside alkylated in the base was eluted in the form of two fractions. It was possible to elucidate the actual compositions of these fractions when the reaction mixtures were separated by ion-exchange and reversed-phase HPLCs.

The results of the separation by ion-exchange HPLC of the products of the alkylation of AMP by thiotepa in a neutral medium (more accurately, in the absence of a proton donor) are shown in Fig. 2a. The first fraction (A), containing adenosine,

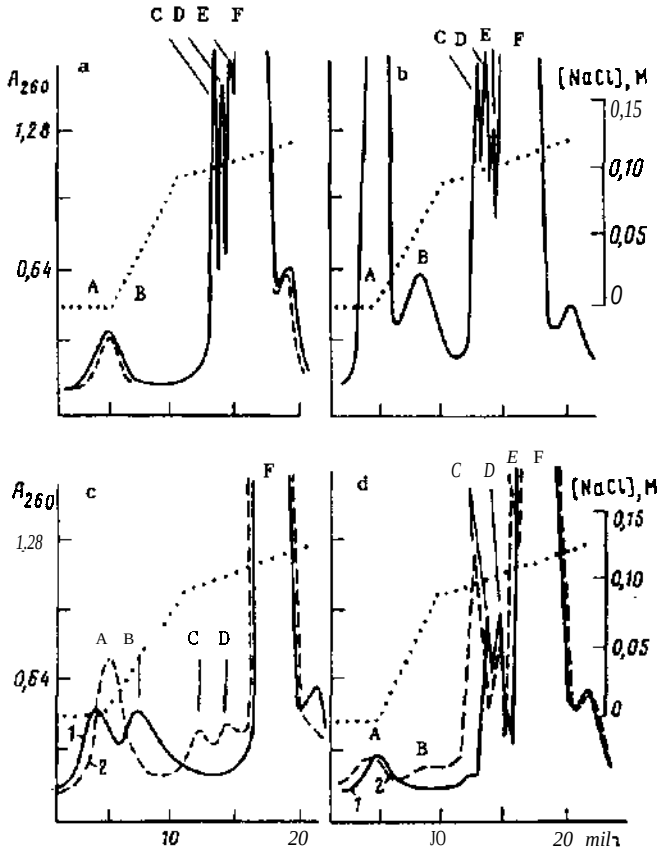


Fig. 2. Ion-exchange HPLC on a Bio-Gel TSK DEAE-5-PW column (7.5 x 75 mm) in a concentration gradient of NaCl of the products of the alkylation of nucleotides. The number of the curve, the nucleotide + agent + proton donor (if used) (their ratio), and the pH are given; a)–, AMP + thiotepa (1:2), 7; b) -, AMP + thiotepa + $HClO_4$ (1:1:1), 4.5; c) 1, AMP + ethyleneimine (1:2), 9.5-10; c) 2, dCMP + thiotepa (1:4), 7.0; d) 1, GMP + thiotepa (1:1), 7.0; d) 2, AMP + ethyleneimine + $HClO_4$ (1:2:2), 7.0.

and the last (in the figure they are indicated by broken lines) were present in the initial AMP, while the three fractions issuing before the AMP (C, D, and E) were alkylation products. Acidification of the medium to pH 4.5 (alkylation by the immonium cation [8]) led not only to an increase in the total yield of reaction products but also to the appearance of the new fraction B (Fig. 2b). It is striking that the general pattern of separation of the mixture in this figure coincides almost completely with that shown in Fig. 1 (curve 1), with the exception of fraction E, which was separated better in the case of HPLC.

On the acid hydrolysis of the product from fraction A of Fig. 2b (1 N HC1, 100°C, 1 h) and analysis of the reaction products by reversed-phase HPLC, free adenine and its substituted analogues were detected, the amount of alkyladenines being 2-3 times greater than that of the unsubstituted base (Fig. 3). A similar chromatogram was obtained for a hydrolysate of fraction B, differences consisting in a larger amount of N^6-substituted adenine and the absence of unsubstituted adenine. The appearance of products of N^6-substitution in reactions with an initial pH of 4.5 was apparently connected with a rise of the pH to neutrality in the course of the reaction, when the $N1 \rightarrow N^6$ rearrangement became possible.

On the alkylation of AMP by ethyleneimine (Fig. 2c, curve 1), fraction A remained the same as in the initial nucleotide, and, consequently, fraction B was nothing other than aminoethylated adenosine partially retained by the column because of the high basicity of the primary amino group in the alkyl radical.

We therefore assume that the appearance of fraction B in the case of the alkylation of AMP by thiotepa (Fig. 2b) was due to the presence of perchloric acid in the reaction mixture, leading to the hydrolysis of the phosphoramide bonds of the

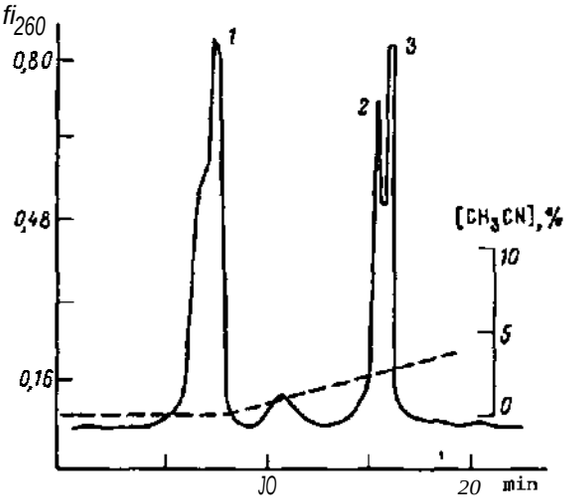


Fig. 3. HPLC of a hydrolysate of fraction A in Fig. 2b on a Bio-Sil ODS-5S column in a concentration gradient of acetonitrile in 0.05 M sodium phosphate buffer, pH 7.0. 1) 1-R-Ade; 2) N^6-R-Ade; 3) Ade.

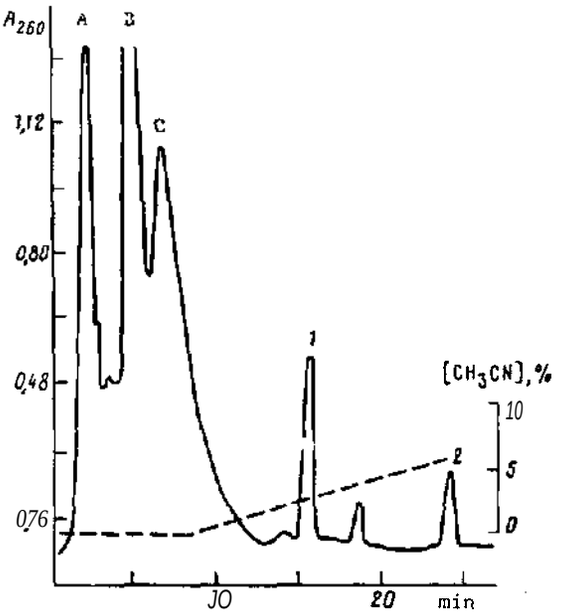


Fig. 4. HPLC on a Bio-Sil ODS-5S column of the products of the alkylation of AMP by ethyleneimine in an alkaline medium in the absence of a proton donor. 1) R-Ado; 2) Ado.

alkylating agent and to the formation of aminoethyl derivatives, since no such fraclion was formed in the reaction without the acid (Fig. 2a).

On the alkylation of AMP by ethyleneimine (pH of the medium 9.5-10) in the absence of $HClO_4$, fractions C-E were not detected (Fig. 2c, curve 1). We explain this by the assumption that the aminoethyl residues that had entered the AMP molecule gave rise to an affinity of the modified nucleotides for the anion-exchange resin, thanks to which they were eluted from the column together with the unmodified AMP. This was confirmed in experiments on the separation of such mixtures in the

regime of reversed-phase HPLC. The fraction in Fig. 2c corresponding to the unmodified nucleotide (curve 1) was inhomogeneous and contained, in addition to AMP, not less than two alkylation products (Fig. 4). The products of this chromatographic procedure were not studied in detail, but acid hydrolysis of the fractions showed that one of them (A) gave as the main component of the hydrolysate the N^6-alkyladenine, while two others (B and C) gave adenine; one of these fractions was. therefore, the nucleotide alkylated in the phosphate group. AMP alkylated by ethyleneimine in the presence of $HClO_4$ (pH 7), when the aminoethyl group was present in the salt form ($-NH_3ClO_4^-$), revealed on a chromatogram the same fractions as AMP alkylated by thiotepa. Here, obviously, compound (II) was eluted in fraction D, and compound (IV) in fraction C.

In neutral and weakly acidic media, the alkylation of the other nucleotides that we studied — ribo- and deoxyribonucleoside 5′-phosphates (GMP, dCMP, TMP, and UMP) — took place in a similar way to that of AMP. Chromatograms of the separation of the products of the alkylation of dCMP and GMP by thiotepa in a neutral medium are given in Figs. 2c (curve 2) and 2d (curve 1). A difference that is characteristic for nucleotides of the pyrimidine series is the absence of fraction E (Fig. 2c, curve 2); in our opinion, this is due to the following circumstances.

It can be seen from a comparison of fractions C and E, alkylated in the base, that alkylation only in the base took place to a smaller degree. Alkylation of the base as a component of a monoanion — i.e., the nucleotide already modified in the phosphate group and having a smaller negative charge on this group — proved to be more effective. This agrees with statements in the literature [11] that alkylation of a base residue in poly(A) takes place 2-3 times more effectively than in the mononucleotide.

From what has been said, it may be assumed that at the very beginning of the reaction the attack of the alkylating agent is directed to the most reactive centers of the nucleotide — the phosphate group in the form of a dianion, and the N7 position of GMP. Then the base residues, now as components of monoanions, are alkylated in accordance with their nucleophilicities. It is just this that explains the absence of fraction E on the alkylation of dCMP, the heterocyclic base of which is less reactive than the purine bases and can take part in the reaction only as a component of a monoanion. A further increase in the reaction time led to the decomposition of the alkylated nucleotides (at the base moieties, in particular), and, for guanine derivatives, even 10 the opening of the imidazole rings. This, in its turn gave an increase in the yield of the alkylated nucleosides forming fractions A and B.

Thus, the results presented show the basic possibility of the alkylation by ethyleneimine and its derivatives of practically all the basic heterocycles of the purine and pyrimidine bases of nucleotides, and the fact that the direction of modification of the bases coincides completely with the direction of the reactions in the corresponding nucleosides [1]. The efficiency of alkylation depends both on the reaction conditions (it rises with the acidification of the medium) and on the reactivities of the base residues, and, for the nucleotides that we have studied, it decreases sharply in the sequence guanine — adenine — cytosine — uracil — thymine. It must be mentioned that in the case of TMP we isolated no alkylation products. The conclusion of the zero or extremely low modification of thymidine 5'-phosphate was made on the basis of a comparison of chromatograms before and after alkylation, which scarcely differed.

EXPERIMENTAL

In this work we used nucleotides from Reanal (Hungary). Thiotepa was obtained by the procedure of [12]. Ethyleneimine of domestic production was redistilled and stored over NaOH. Alkylated mixtures of nucleotides were separated on a column (20 x 30 mm) of cellulose DE-52 that had previously been treated with a solution of ammonium chloride (it was stirred periodically in 2 M NH_4Cl for 2-3 h and was carefully washed with water). Elution from the column was conducted first with water (until UV absorption was absent), and then in a 0.05-0.25 M NaCl gradient (2 x 150 ml), pH 6.0. The HPLC of the alkylated mixtures was conducted on a Bio-Rad instrument (USA) with a flow-through UV detector of the UV monitor, model 1306, type. For separating the hydrolysates in the regime of reversed-phase HPLC we used a 4 x 150 mm Bio-Sil ODS-5S column (Bio-Rad, USA) at a rate of elution of 1 ml/min and a pressure of 80 atm in an acetonitrile – 0.05 M sodium phosphate buffer (pH 7.0) gradient (0-20% of acetonitrile). Ion-exchange HPLC was conducted on a 7.5 x 75 mm Bio-Gel TSK DEAE-5-PW column (Bio-Rad, USA) at a rate of elution of I ml/min and a working pressure of 25 atm in a concentration gradient of NaCl (0.005-1.0 M), pH 6.0. Separation by the TLC method was conducted on Silufol UV-254 plates in the alkaline solvent system isopropanol – ammonia (25%) – water (7:1:2). The substances on the plates were detected with the aid of a Khromatoskop-M instrument of domestic production.

NMR spectra were recorded on a Varian Gemini-200 instrument (USA) with working frequencies of 50.4 MHz for ^{13}C nuclei and 199.96 MHz for ^{1}H. PMR spectra were taken in D_2O and ^{13}C NMR spectra in D_2O or DMSO, the internal standard being DMSO-d_6 [sic]. ^{31}P NMR spectra were recorded on a Bruker WP 200 instrument (Germany) with a working frequency of 81.03 MHz, using 83% H_3PO_4 as internal standard.

The hydrolysis of samples taken from the peaks of the curves of the fractions under investigation was carried out in 1 N HCl at 100°C for 1 h.

Alkylation of Nucleotides in the Absence of a **Proton Donor.** A solution of 0.1 mmole of a nucleotide in 0.5 ml of water was added to a solution of 0.2 mmole of the alkylating agent in 1.5 ml of water. The mixture was kept at 37°C for 12-16 h or at 20°C for 1 day. After the extraction of the unchanged alkylating agent with ether or chloroform, the mixture was deposited on a column. The weight of sample for HPLC was 2 mg, and for ion-exchange chromatography on cellulose it was 30 mg.

Alkylation of Nucleotides in a Weakly Acid **or Neutral Medium.** The ratio of the reactants was changed or the pH of the medium was adjusted by the addition of dilute $HClO_4$ solution according to the requirements of the experiment (see the captions to the figures). For details of alkylation in the presence of $HClO_4$ (by the immonium cation), see [8].

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