The Ethylation of Nucleic Acid-Bases with Triethyl Phosphate

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Uracil, thymine, cytosine, adenine, and guanine were alkylated by triethyl phosphate in a homogeneous aqueous phase at 60—80 °C, giving the corresponding ethyl derivatives in considerable yields. The reactivity order on the ethylating site in each base was found to be as follows; uracil, thymine, and cytosine: N-1>N-3; adenine: N-9≈N-3>N-7, N-1; guanine: N-1>N-7>N-3, N-9.

It has been known that the structural deformation of nucleic acids caused by such alkylating agents as mustards may be relevant to their mutagenic and carcinogenic effects on many living systems. Therefore, the action of various alkylating agents, such as dialkyl sulfates,[1-5] alkyl alkanesulfonates,[3-6] alkyl halides,[5-8] diazoalkanes,[9] and mustards,[10] on nucleic acids and their components has been studied actively.

In previous papers, we reported that trialkyl phosphates were very useful for the *N*-alkylation of nitrogen heterocycles, such as imidazoles,[11,12] pyrimidines,[13] and purines.[14,15] Especially, trimethyl and triethyl phosphates are suited for the alkylation of many natural products because of their moderate reactivity and high solubility in water. In practice, the methylations of nucleic acid-bases with trimethyl phosphate have been carried out successfully in a homogeneous aqueous phase.[16]

While many methylation studies of nucleic acids and their components have been reported, little is known about ethylation. The ethylating reaction has though been reported to show mutagenic effects in some biological systems, and Singer *et al.* indicated that ethyl ethanesulfonate and ethyl methanesulfonate were good mutagenes.[17] Furthermore, Kononova and Gumanov reported that triethyl phosphate (TEP) inactivated phage T4V more readily than did trimethyl phosphate (TMP), although TMP was more mutagenic.[18] Industrially, TEP has been employed as a gasoline additive and a flame retardant for polymers and paints; in addition, TEP has even been proposed as a food additive.

In this regard, we have investigated the ethylation of nucleic acid-bases, such as uracil (**1**), thymine (**2**), cytosine (**3**), adenine (**11**), and guanine (**12**) with TEP in an aqueous phase.

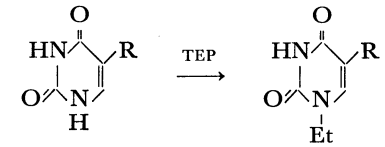
Below we will characterize the products from these reactions. The reaction sites on ethylation will also be compared with those on methylation.

Results and Discussion

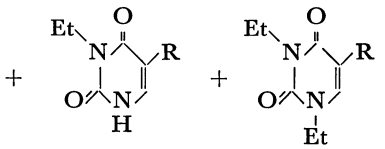
The reactions were carried out at 60—80 °C by stirring a mixture of a base and a 2—5 molar excess of TEP in water. (The pH values were 10—10.5 for **1**, **2**, **3**, and **11** and pH 12 for **12**. A relatively high pH value was used for the reaction of **12** in order to obtain a homogeneous solution.) The yields of the products were determined by means of the UV spectra. The products were isolated through extraction and column chromatography and were identified conveniently by means of their UV and NMR spectra. The results are summarized in Tables 1 and 2.

Uracil (1), Thymine (2), and Cytosine (3). Compounds **1** and **2** are generally alkylated with alkyl halide to give 1-alkyl and 1,3-dialkyl derivatives.[19,20] Diazomethane also alkylated **1** to give the corresponding *N*-methyl and *O*-methyl derivatives.[21] We reported previously that trimethyl phosphate (TMP) alkylated these pyrimidines at the N-1 and N-3 positions in almost equal amounts to give 1-methyl, 3-methyl, and 1,3-dimethyl derivatives.[16]

On the other hand, the present reactions of **1** and **2** with TEP gave results different from those of methylations, showing ethylation to take place mainly at the N-1 position. Thus, the reactions of **1** and **2** with TEP afforded the corresponding 1-ethyl derivatives (**4** and **7**) chiefly, along with 3-ethyl derivatives (**5** and **8**) in small yields. When the reaction temperature was raised to 80 °C, 1,3-diethyl derivatives (**6** and **9**) were also produced.



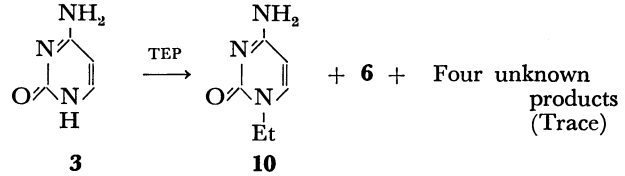
1: R=H, **2**: R=Me — **4**: R=H, **7**: R=Me



5: R=H, **8**: R=Me — **6**: R=H, **9**: R=Me

In cytosine (**3**), dimethyl sulfate in DMF has been reported to methylate **3** at the N-3 position mainly to give 3-methylcytosine,[1] while TMP has been shown to methylate **3** at the N-1 position predominantly to give 1-methylcytosine.[16]

The present reaction of **3** with TEP at 80 °C afforded six UV absorbing products (**6**, **10**, and four unknown products). Compound **10** was produced mainly and



3 — **10**

was identified as 1-ethylcytosine through its physical constants. Compound **6** was produced in a small yield and was identified as 1,3-diethyluracil through its UV

TABLE 1. REACTIONS OF NUCLEIC ACID-BASES WITH TRIETHYL PHOSPHATE (TEP)[a]

Base	pH	Product	Yield (%)			UV Spectra λ_{max}		
			60 °C, 48 h	80 °C, 24 h	80 °C, 48 h	pH 1	pH 7	pH 13
Uracil (U)	10—10.5	1-Ethyl-U	15	23	34	267.0	268.0	268.0
		3-Ethyl-U	6	8	10	260.0	261.0	284.0
		1,3-Diethyl-U	0	5	7	267.0	268.0	268.0
Thymine (T)	10—10.5	1-Ethyl-T	14	24	34	273.0	273.0	271.0
		3-Ethyl-T	6	9	12	260.0	267.0	290.0
		1,3-Diethyl-T	0	4	6	273.0	272.0	273.0
Cytosine (C)	10—10.5	1-Ethyl-C	13[b]	15	17	284.0	276.0	276.0
		1,3-Diethyl-U	0	2	3			
		Unknown A	0	Tr[c]	Tr	261.0	271.0	271.0
		Unknown B	0	Tr	Tr	267.0	265.0	269.0
		Unknown C	0	0	Tr		270.0	
		Unknown D	0	0	Tr		277.0	
Adenine (A)	10—10.5	9-Ethyl-A	26[b]	33	39	261.0	263.0	263.0
		3-Ethyl-A	15[b]	22	29	275.0	275.0	274.0
		7-Ethyl-A	0	4	6	273.0	269.0	269.0
		N^6, 9-Diethyl-A	0	1	3	265.0	268.0	267.0
		Unknown E	0	Tr	Tr	268.0	268.0	270.0
		Unknown F	0	0	Tr	263.0	263.0	268.0
Guanine (G)	11—12	1-Ethyl-G	0	37	28	249.5 273.5s)	248.0 272.0s)	
		7-Ethyl-G	5[b]	12	17	251.0 275.0s)	250.0s) 282.5	280.0
		Other minor products	—	Tr	Tr		[d]	

a) The ratio of TEP to base was 6 : 1 at 80 °C and 3 : 1 at 60 °C. b) Isolated yield. c) Tr refers to a trace yield. d) The yields of these minor products were too small for the UV spectra to be measured. s) Shoulder.

TABLE 2. REACTIONS OF ALKYLATED NUCLEIC ACID-BASES WITH TRIETHYL PHOSPHATE (TEP)

Base	pH	TEP/Base	Temp (°C)	Product	Yield (%)[a]	
					24 h	48 h
1-Ethyluracil	10	6	80	1,3-Diethyluracil	23	28
1-Ethylthymine	10	6	80	1,3-Diethylthymine	16	22
1-Methylcytosine	10	6	80	3-Ethyl-1-methyluracil	8	13
9-Ethyladenine	10	6	80	N^6,9-Diethyladenine	4	10

a) Spectroscopic yields.

spectrum. Although 1,3-diethylcytosine was not observed in the reaction mixture, the formation of **6** may be attributed to the rapid alkaline hydrolysis of 1,3-diethylcytosine. In practice, the analogous compound, 1,3-dimethylcytosine, was converted to 1,3-dimethyluracil under similar conditions. The UV spectra of all possible alkyl derivatives of **3** were compared with those of the four unknown products (A-D), but only one compound showed an UV spectrum similar to that of O^2-methylcytosine. Although the alkylation of the cytosine ring at the O^2-atom has not been reported, Singer *et al.* have suggested the possibility of the formation of O^2-ethylcytidine from the reaction of cytidine with ethyl iodide.[5] When the reaction temperature was lowered to 37—60 °C, **10** was formed as the sole product.

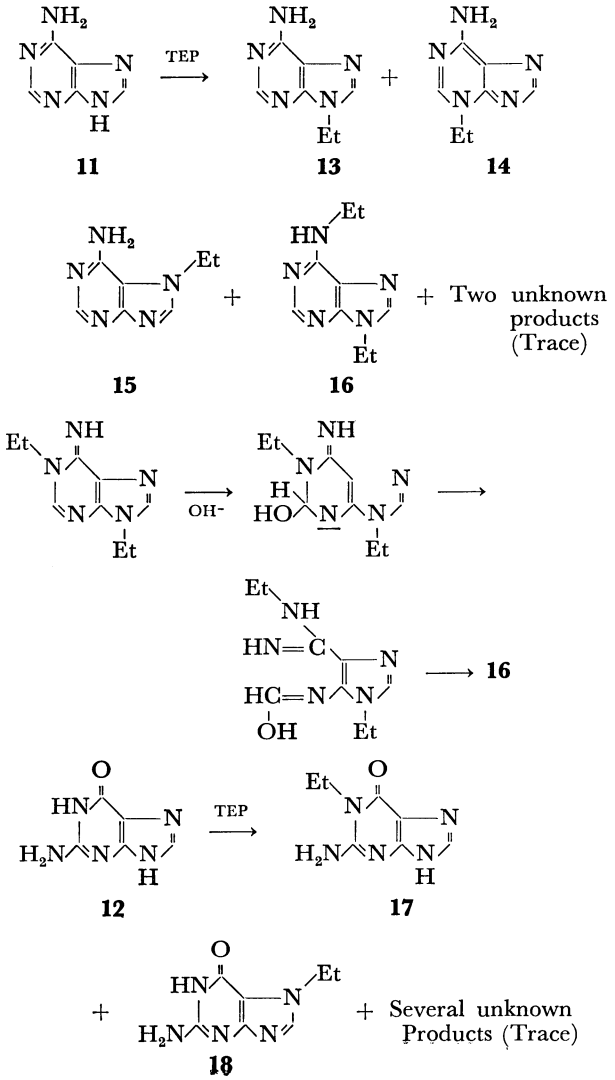
The difficulty of ethylation at the N-3 position of these three pyrimidine bases (**1**, **2**, and **3**) with TEP might be attributed to the steric hindrance on the approach of TEP to the N-3 position by two adjacent carbonyl groups of **1** and **2** and amino and carbonyl groups of **3**. Similar results were shown in the alkylation of xanthine and its derivatives.[15] However, when the reactions were performed at 80 °C, 1-alkylpyrimidines (**4**, **7**, and 1-methylcytosine) reacted with TEP to afford the corresponding 3-ethyl derivatives.

Since the rates of the reactions of **1**, **2**, and **3** were enhanced with the increase in the pH value of the reaction mixture, **1**, **2**, and **3** may react in the anionic form with TEP. The slower reaction of **3** than **1** and **2** may, therefore, be originated by the larger pK_a value of **3** (12.15) than those of **1** (9.46) and **2** (9.90).

Adenine (11) and Guanine (12). These purine bases of nucleic acids have been known to be reactive towards alkylating agents. The alkylations of **11** with various alkylating agents have been studied actively,

and, thanks to these works, **11** has been shown to be alkylated at the N-3 position under neutral conditions,[2,4,22] while under basic conditions the N-9 position was alkylated mainly, with the co-formation of N-3 substituted adenine.[8] However, a reaction of **11** with TMP in a homogeneous alkaline aqueous phase afforded 3-methyl- and 9-methyladenines chiefly in almost equal yields, with the co-formation of small amounts of N^6-methyl- and 7-methyladenines.[16]

The present reaction of **11** with TEP at 80 °C gave six UV absorbing products. Two main products (**13** and **14**) were isolated and identified as 9-ethyl- and 3-ethyladenines respectively through their physical properties. The yield of **13** was a little more than that of **14**. Compounds **15** and **16** were produced in low yields and were identified as 7-ethyl- and N^6,9-diethyladenines through their UV spectra which corresponded excellently to those of 7-methyl- and N^6,9-dimethyladenines. N^6,9-Dialkyladenine was established to be formed by the Dimroth rearrangement of 1,9-dialkyladenine under alkaline conditions.[16] When the reaction temperature was lowered to 37—60 °C, only two products, **13** and **14**, were formed. These results show that the reactivity order of the four nitrogens of **11** is similar to that in the methylation, that is, N-9≈N-3>N-7, N-1.



Although **13**, which is considered as the model of adenosine, was not as reactive as adenine itself, **13** was ethylated with TEP at 80 °C at the N-1 position, which has been established as the most nucleophilic and basic site in 9-alkyladenine. Resulting 1,9-diethyladenine was rearranged *in situ* to give N^6,9-diethyladenine.

On the other hand, only a few studies have been carried out on the alkylation of guanine (**12**); with ethyl ethanesulfonate[4] and dimethyl sulfate,[2] the formation of 7-ethyl- and 7,9-dimethylguanines has been reported. Recently, we reported that **12** was successfully methylated with TMP in water at pH 11—12 to give 1-methylguanine mainly, with the co-formation of 3-methyl-, 7-methyl-, and O^6-methylguanines.[16]

The present reaction of **12** with TEP at 80 °C afforded several UV-absorbing products; the two main products could be identified as 1-ethyl- and 7-ethylguanines (**17** and **18**) through their UV spectra. Compound **17** was produced chiefly at 80 °C, but, at 60 °C, **18** was given as the sole product. Since **12** was consumed for the ethylation reaction in a rate similar to that in the ethylation of **11**, the reactivity of **12** towards TEP may be considered to be almost the same as that of **11**. Compound **17** gradually decreased in quantity with the progress of reaction time; probably it was subsequently ethylated to diethylguanine, which, however, could not be isolated.

Conclusion

TEP was found to react with five nucleic acid-bases in a homogeneous aqueous phase because of its solubility in water. Although TEP was not so reactive as TMP, which reacted with five nucleic acid-bases at 25—37 °C very well, at 60—80 °C five nucleic acid-bases were ethylated with TEP, giving the corresponding ethyl derivatives in yields comparable with those obtained by the use of other ethylating agents.

The reactivity order of five nucleic acid-bases towards TEP was found to be as follows, based on the consumption of the starting materials of the reaction with TEP: adenine (**11**)≈guanine (**12**)>uracil (**1**)≈thymine (**2**)>cytosine (**3**). In each base, ethylation occured in the following order: uracil, thymine, and cytosine: N-1>N-3; adenine: N-9≈N-3>N-7, N-1; guanine: N-1>N-7>N-3, N-9. This reactivity order of bases and sites in each base was almost the same as in the methylation. However, the sterically hindered sites, the N-3 positions of **1**, **2**, and **3**, were less reactive in ethylation than in methylation.

The alkylated bases shown in Table 2, which have alkyl groups in the same position as nucleosides have ribose or deoxyribose, also reacted with TEP to afford the dialkyl derivatives. The reactivity order of these alkylated bases was different from that of the parent bases: 1-ethyluracil (**4**)>1-ethylthymine (**6**)>1-methylcytosine>9-ethyladenine (**13**).

Thus, TEP has a considerable ethylating ability of the base moiety of nucleic acids, therefore, it seems reasonable to pay attention to the use of TEP as an additive for commercial products.

Experimental

The UV and IR spectra were measured with Hitachi EPS-3T and Jasco IR-G spectrometers respectively. The

NMR spectra were recorded on a Hitachi-Perkin Elmer R-20 spectrometer, with a dilute solution in deuterioxide, deuteriochloroform, or trifluoroacetic acid and tetramethylsilane as an internal or an outside standard. Thin-layer chromatography was performed on silica gel [GF_{254} (type 60), Merck] or aluminium oxide [PF_{254} (type 150), Merck] or cellulose [13254, Eastman] using a mixture of chloroform and methanol in the following volume ratio. Solvent A: 10 : 1, B: 5 : 1, C: 5 : 2, or Solvent D: 1-propanol-concentrated ammonium hydroxide-water, 9 : 1 : 3. Column chromatography was carried out using silica gel (Merck, Art. 7734, 70—230 mesh) or cellulose (CF 11, Whatman).

Commercially available uracil (**1**), thymine (**2**), cytosine (**3**), adenine (**11**), and guanine (**12**) as well as TEP were used without further purification.

*Ethylation of Uracil (**1**).* *A*: A mixture of **1** (112 mg, 1.0 mmol) and TEP (1.07 g, 6.0 mmol) in water (2.0 ml) was stirred at 80 °C. The solution was maintained at pH 10—10.5 throughout the reaction by the occasional addition of 4M sodium hydroxide. After stirring for 48 h, 3 μl of the reaction mixture was spotted in aluminum oxide thin-layer chromatography. The plate was developed immediately using Solvent A. Three UV-absorbing products (**4**, **5**, and **6**) were observed. (R_f value; **1**: 0.03, **4**: 0.35, **5**: 0.56, **6**: 0.95). Each spot was then scraped individually from the plate to extract the substance on the spot with 4 ml of water. The yield of each product was calculated, by means of a procedure similar to that described in a previous paper,[16] from the absorbancy of the solution; we thus found the yields of **4**, **5**, and **6** were 34, 10, and 7% respectively.

B: A similar reaction of **1** (1.21 g, 10.8 mmol) and TEP (5.35 g, 2.90 mmol) at 60 °C was carried out. After stirring for 48 h, the reaction mixture was neutralized with concentrated hydrochloric acid to give a precipitate of **1**. The mother liquor was then extracted with chloroform. After the solvent had then been removed from the organic layer, the addition of hexane to the residue afforded the crystals of 1-ethyluracil (**4**, 0.05 g, 4%); mp 146—147.5 °C (from THF-ether) (lit,[19] mp 147.5 °C); NMR ($CDCl_3$): 1.28 (t, 3H, $-CH_2CH_3$, J=7 Hz), 3.77 (q, 1H, $-CH_2CH_3$, J=7 Hz), 5.67 (d, 1H, **H**^{5}, J=8 Hz), 7.18 (d, 1H, **H**^{6}, J=8 Hz), and 10.17 ppm (δ) (bs, 1H, N^3-**H**).

*Ethylation of Thymine (**2**).* *A*: The treatment of **2** (126 mg, 1.0 mmol) with TEP (1.07 g, 6.0 mmol) in water (2.0 ml) at 80 °C for 48 h gave 1-ethylthymine (**7**, 34%), 3-ethylthymine (**8**, 12%), and 1,3-diethylthymine (**9**, 6%), whose yields were calculated in a way similar to that used in the ethylation of **1**. (R_f value on aluminumn TLC using Solvent A; **2**: 0.06, **7**: 0.49, **8**: 0.65, **9**: 0.98)

B: The isolation of 1-ethylthymine (0.08 g, 5%) was carried out in a manner similar to that used for 1-ethyluracil after a mixture of **2** (1.30 g, 10.30 mmol) and TEP (5.35 g, 29.0 mmol) in water (10 ml) had been stirred for 48 h at 60 °C; mp 220—222 °C (from THF-ether) (lit,[23] mp 223 °C); NMR ($CDCl_3$) 1.42 (t, 3H, $-CH_2CH_3$, J=7 Hz), 2.02 (d, 3H, $-CH_3$, J=1.2 Hz), 3.88 (q, 2H, $-CH_2CH_3$, J=7 Hz), 7.09 (d, 1H, **H**^{6}, J=1.2 Hz), and 9.90 ppm (δ) (bs, 1H, N^3-**H**).

*Ethylation of Cytosine (**3**).* *A*: A mixture of **3**(111 mg, 1 mmol) and TEP (1.07 g, 6.0 mmol) in water (2 ml, pH 10—10.5, NaOH) was stirred at 80 °C for 48 h. The reaction mixture (4 μl) was then developed on a silica gel TLC using Solvent C, giving the following spots. R_f value; **3**: 0.18, **6**: 0.76, **10**: 0.45, unknown A: 0.73, B: 0.57, C: 0.49, D: 0.23. A treatment similar to that used in the ethylation of **1** showed that the yields of **10** and **6** were 17 and 3% respectively.

B: A mixture of **3** (1.0 g, 9.0 mmol) and TEP (5.25 g, 29.0 mmol) in water (15 ml, pH 10, NaOH) was stirred at 60 °C for 48 h. The subsequent neutralization of the reaction mixture with concentrated hydrochloric acid gave the precipitate of **3**. After the precipitate had been filtered out, the mother liquor was extracted with chloroform to remove the unreacted TEP. The water layer was concentrated to give a residue, which was subsequently mixed with ethanol to remove any undissolved substances. The residue which was obtained after concentrating the alcoholic solution was treated by silica gel column chromatography (1.5×60 cm). Elution with Solvent C provided the salt of 1-ethylcytosine with diethyl hydrogen phosphate (0.54 g). The salt was subsequently treated with an anionic exchange resin (Dowex 1×8, 200—400 mesh, OH form). Elution with water gave the free form of 1-ethylcytosine (**8**, 0.16 g, 13%); mp 236—238 °C (sublime) (lit,[24] 245—246 °C); NMR (D_2O): 1.22 (t, 3H, $-CH_2CH_3$, J=7 Hz), 3.76 (q, 2H, $-CH_2CH_3$, J=7 Hz), 5.95 (d, 1H, **H**^{5}, J=8 Hz), and 7.58 ppm (δ) (d, 1H, **H**^{6}, J=8 Hz); Found: C, 51.75; H, 6.31; N, 30.51%, Calcd for $C_6H_9N_3O_1$: C, 51.78; H, 6.52; N, 30.20%.

*Ethylation of Adenine (**11**).* A: Compound **11** (135 mg, 1 mmol) and TEP (1.07 g, 6.0 mmol) were stirred in water (2 ml, pH 10—10.5, NaOH) for 48 h at 80 °C. The reaction mixture (4 μl) was then developed on silica gel TLC, using Solvent B, to give the following spots. R_f value; **11**: 0.32, **13**: 0.57, **14**: 0.42, **15**: 0.23, **16**: 0.66, unknown E: 0.10, F: 0.14. Calculations from the absorbancy of the solution of each spot similar to that described above showed the yields of **13**, **14**, **15**, and **16** to be 39, 29, 6, and 3% respectively.

B: A mixture of **11** (1.35 g, 10.0 mmol) and TEP (5.25 g, 29.0 mmol) was stirred in water (10 ml, pH 10, NaOH) at 60 °C for 48 h. The reaction mixture was then neutralized with concentrated hydrochloric acid and extracted with hexane to remove any unreacted TEP. The water layer was concentrated to give a residue, which was subsequently mixed with ethanol to remove any undissolved substances. The residue which was obtained after concentrating the alcoholic solution was then treated by silica gel column chromatography (1.5×60 cm). Elution with Solvent B provided 9-ethyladenine (**12**, 0.42 g, 26%) and then 3-ethyladenine (**13**, 0.24 g, 15%); **12**: mp 202—203 °C (from ethanol) (lit,[25] mp 194—195 °C); NMR ($CDCl_3$): 1.58 (t, 3H, $-CH_2CH_3$, J=7 Hz), 4.25 (q, 2H, $-CH_2CH_3$, J=7 Hz), 6.39 (bs, 2H, $-NH_2$), 7.79 (s, 1H, **H**^{2}), and 8.32 ppm (δ) (s, 1H, **H**^{8}); **13**: mp 233—234 °C (from ethanol) (lit,[26] mp 233 °C); NMR (D_2O): 1.34 (t, 3H, $-CH_2CH_3$, J=7 Hz), 4.13 (q, 2H, $-CH_2CH_3$, J=7 Hz), 7.67 (s, 1H, **H**^{2}), and 7.95 ppm (δ) (s, 1H, **H**^{8}).

*Ethylation of Guanine (**12**).* *A*: After **12** (151 mg, 1 mmol) had been dissolved in water (2 ml, pH 13) at 80 °C, TEP (1.07 g, 6.0 mmol) was added. Soon after the reaction began, the pH of solution dropped to 11—12; this value was maintained by the occasional addition of 4M sodium hydroxide. The reaction mixture (4 μl) was developed on silica gel TLC, using Solvent A, to give the following spots: R_f value; **12**: 0.00, **17**: 0.21, **18**: 0.24, six minor unknown products: 0.03, 0.12, 0.32, 0.35, 0.45, 0.74. After a treatment similar to that described above, the yields of **17** and **18** were found to be 28 and 17% respectively.

B: A mixture of **12** (1.25 g, 8.30 mmol) and TEP (5.25 g, 29 mmol) in water (50 ml, pH 11—12, NaOH) was stirred for 48 h at 60 °C. The reaction mixture was then extracted with chloroform to remove any unreacted TEP. The water layer was concentrated to give a residue, which was subsequently treated by a cellulose column chromatography (2.5×60 cm).

Elution with Solvent D provided a viscous liquid which was crystalized by the addition of a small amount of water and identified as 7-ethylguanine through its UV spectra (**18**, 0.075 g, 5%); mp>300 °C dec; NMR (CF_3CO_2H): 1.14 (t, 3H, $-CH_2C\mathbf{H}_3$, J=7 Hz), 4.15 (q, 2H, $-C\mathbf{H}_2CH_3$, J=7 Hz), and 8.34 ppm (δ) (s, 1H, $\mathbf{H}^8$).

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