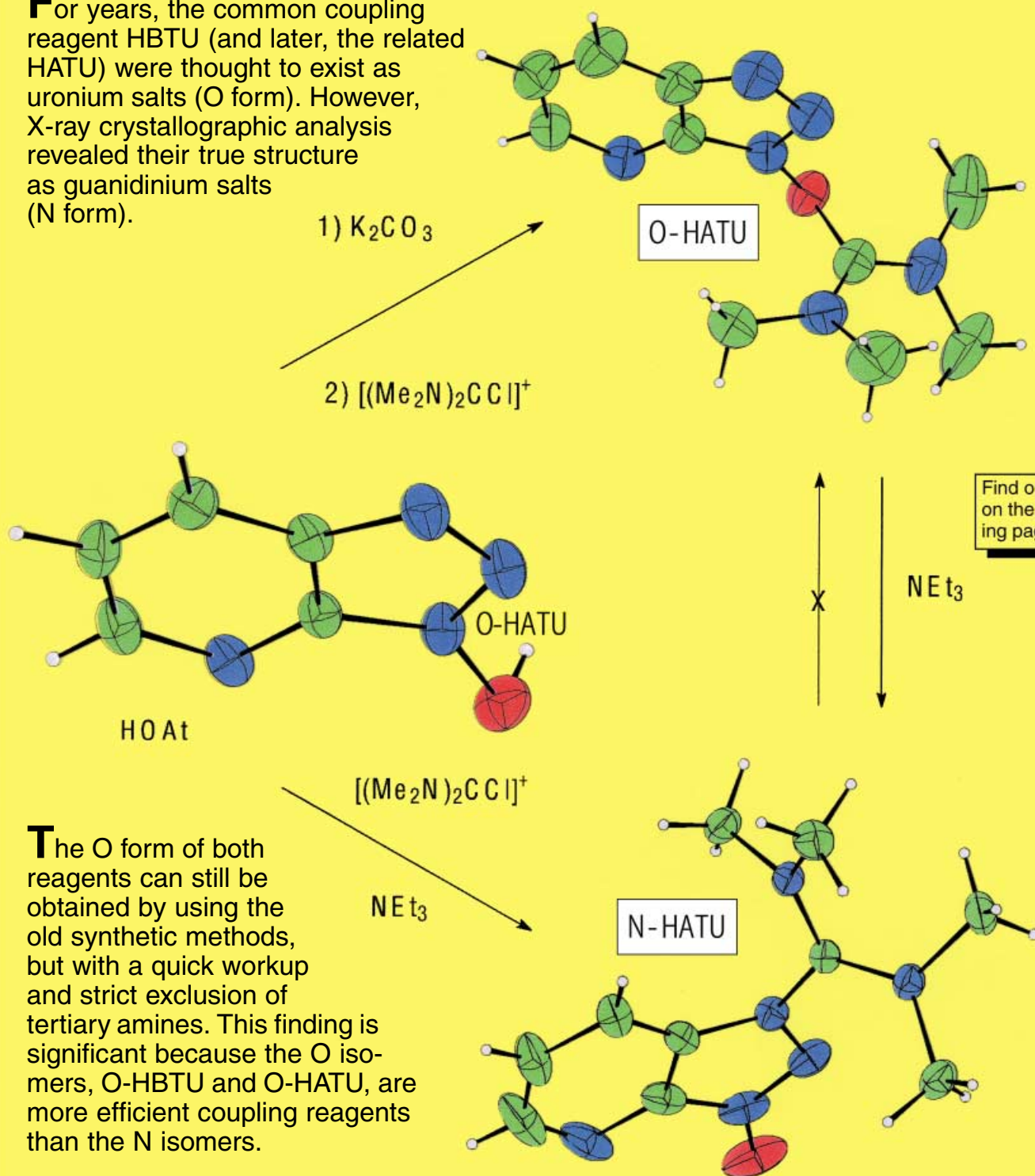


For years, the common coupling reagent HBTU (and later, the related HATU) were thought to exist as uronium salts (O form). However, X-ray crystallographic analysis revealed their true structure as guanidinium salts (N form).

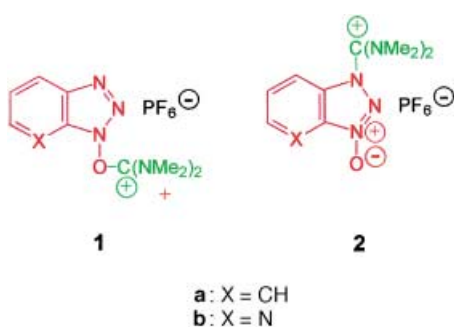


The O form of both reagents can still be obtained by using the old synthetic methods, but with a quick workup and strict exclusion of tertiary amines. This finding is significant because the O isomers, O-HBTU and O-HATU, are more efficient coupling reagents than the N isomers.

The Uronium/Guanidinium Peptide Coupling Reagents: Finally the True Uronium Salts**

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Among the currently most popular peptide coupling reagents are the phosphonium^[1,2] (BOP, PyBOP, etc.) and guanidinium^[3] (HBTU, HATU, etc.) salts. When first described in 1978, HBTU was assigned the structure of uronium salt **1a**, presumably by analogy with the previously described phosphonium salts. In 1993, we synthesized HATU by using the same method,^[4] and the analogous structure **1b** was assigned, in spite of the fact that ¹H NMR spectroscopic data



cited in both papers, viewed in the light of current knowledge,^[5] might have suggested otherwise. A year later, X-ray crystallographic analysis and solution and solid-state NMR

spectroscopy showed that the true structures in both the crystalline state^[6] and in solution^[7,8] were the guanidinium isomers **2** and not the uronium isomers **1**. These results are in agreement with expected thermodynamic stabilities.^[9]

By substituting KOBt^[10] and KOAt^[13] for HOBt and HOAt, and working up the reaction mixture quickly, we have now succeeded in preparing both these compounds in the originally postulated uronium form. These results are of special interest in view of the fact that the new uronium salts are more efficient coupling reagents than the corresponding guanidinium isomers. Although first examined in the case of a methyl-substituted HATU derivative^[8] obtained from 4-Me-HOAt in which the methyl group interferes with the stability of the N-form, this structural element is not essential if attention is paid to the ease of isomerization of the O- to N-form by organic bases such as triethylamine. This process was easily followed in the IR spectrum (see below). The reverse rearrangement has never been observed. X-ray crystallography^[14] was used to confirm that both HBTU and HATU obtained from the potassium salts are in the O-form (Figure 1).

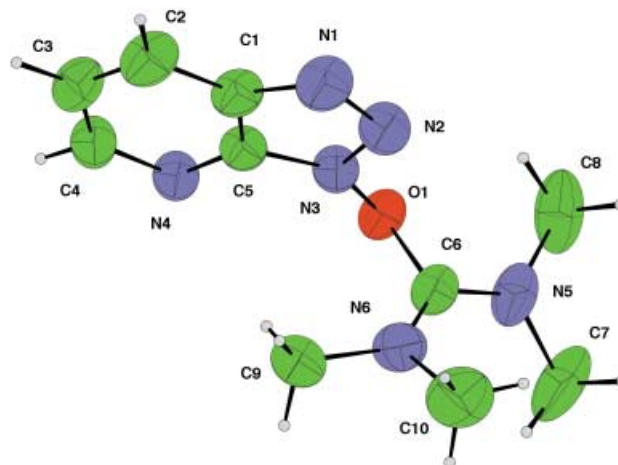


Figure 1. Crystal structure of **1b**.

Once several uronium compounds had been synthesized and their structures established by means of X-ray crystallographic analysis, we were able to find characteristic spectral absorptions that now easily allow structural assignment on the basis of IR, ¹H, and ¹³C NMR analysis (Table 1). The IR spectra of salts derived from tetramethylurea showed characteristic absorptions at 1709–1711 for the O-isomers and at 1664–1675 cm⁻¹ for the N-derivatives. The twelve dimethylamino protons of the O-derivatives give rise to a singlet near $\delta = 3.24$ in the ¹H NMR spectrum, whereas the N-compounds showed two singlets for six protons each near $\delta = 3.0$ and 3.4. These differences in the proton NMR spectra agree with expectations based on the effect of hindered rotation in related systems.^[5]

As expected (C–O vs C–N bond breaking),^[15] amino acid activation through the authentic uronium salts proceeded more rapidly than through the isomeric guanidinium species under reaction conditions that avoided prior O → N rearrangement. Thus, Z-Aib-OH was converted into Z-Aib-OAt

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Supporting information for this article is available on the WWW under <http://www.angewandte.com> or from the author.

Table 1. IR, ¹H, and ¹³C-NMR Absorptions for N- and O-HXTU Species^[a]

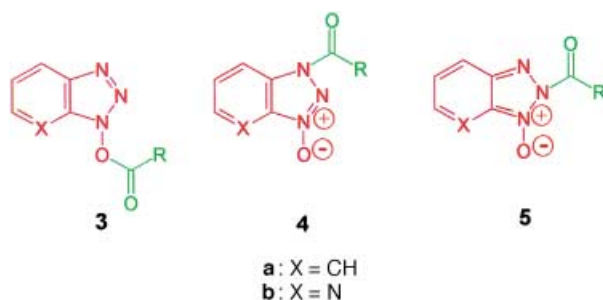
Guanidinium Type				Uronium Type			
	IR [cm ⁻¹]	¹ H-NMR ^[b]	¹³ C-NMR ^[c]		IR [cm ⁻¹]	¹ H-NMR ^[b]	¹³ C-NMR ^[c]
N-HATU	1668.9	3.02s, 3.37s	151.9	O-HATU	1711.5	3.24s	162.2
N-4-Me-HATU	1670.4	3.02s, 3.48s	150.1	O-4-Me-HATU	1711.5	3.25s	161.9
N-4-Cl-HBTU	1675.6	3.00s, 3.44s	149.3	O-4-Cl-HBTU	1711.1	3.21s	161.7
N-HBTU	1664.4	3.02s, 3.37s	152.7	O-HBTU	1709.3	3.21s	162.0

[a] IR and NMR data were obtained in CH₃CN and CD₃CN, respectively. [b] Methyl protons. [c] Cationic carbon atom.

with O-HATU in the presence of one equivalent of collidine (half-life less than 2 min), whereas with N-HATU under the same conditions $t_{1/2}$ was about 7 min. For HBTU, $t_{1/2}$ was greater than 1 h for both O- (40% OBt ester at 1 h) and N-isomers (29% OBt ester at 1 h). As expected, these differences are reflected in the ease of peptide assembly, for example, in the assembly of the model decapeptide ACP by the so-called "1.5 × 1.5" system.^[16] This represents a model in which a 1.5 molar excess of amino acid and a 1.5 min coupling time brings out the deficiencies of sub-optimal coupling reagents. For manual syntheses with N- and O-HATU, the crude peptide purity was 66.4 and 81.2%, respectively.

In the case of the activation and coupling of peptide segments, the coupling reagent determines the extent of epimerization as well as the yield. The best results were obtained in the presence of TMP base. Thus for the [2+1] coupling in DMF to form Z-Phe-Val-Pro-NH₂,^[17] a well-studied model, the extent of epimerization for O- and N-HATU and O- and N-HBTU in the presence of TMP/DIEA (1:1) was 3.4, 5.9, 10.3, and 20.6, respectively. In contrast, with DIEA, which rapidly effects isomerization, both O- and N-HATU lead to about 10% epimerization.

Our present recognition of the existence of stable O- and N-forms of the uronium/guanidinium type recalls the well-known establishment of the structure of the O- and N-acyl species **3** and **4**, respectively, which are generated during the activation of an acid in the presence of HOXt and a carbodiimide or through HXTU in the presence of a base.



These activated species have been considered to be the key participants in peptide coupling processes that occur in the presence of HOXt since the classical work of König and Geiger^[18] on the value of HOBT as a coupling additive. Initially, in addition to the O-isomer **3a** and the N-isomer **4a**, a second N-acyl species **5a** was postulated. Although X-ray crystallography has been used to establish examples of **3** and **4** in different systems,^[19] unequivocal evidence for structure **5** is lacking.

IR investigations into the activation process show that the O-form **3** is generated rapidly, and is subsequently isomerized to the N-form **4** more or less rapidly, depending on the nature of the system and/or solvent. Since the O-acyl form is more reactive,^[20] such effects may be one factor in rationalizing the greater efficiency of carrying out peptide assembly with "low (short time) preactivation." Examples include the Jung–Redemann decapeptide sequence^[21] in the presence of N-HATU (51.4% yield with a 30-sec preactivation and 30.7% with a 7-min preactivation) and the formation of ACP^[16] with N-TBTU (60.2% yield with a 30-sec preactivation and 34.1% with a 7-min preactivation (see Supporting Information).

A connection can be established between the two varieties of O-species discussed herein, since HPLC analysis allows the separation of the O- and N-acyl derivatives **3a** and **4a** for a number of simple amino acids such as Fmoc-Val-OH.^[22] Thus treatment of Fmoc-Val-OH with O-HBTU in DMF in the presence of one equivalent of DIEA gave O-species **3** (99.5%) and N-acyl derivative **4** (0.5%) within 2 min (88:12 after 15 min). With N-HBTU the O-/N-isomer ratio after 2 min is 87.9:12.1, which suggests a clear relationship between the structure of the coupling reagent and that of the initial activation species. Since the major extent of acylation occurs in the first few minutes of reaction time, these effects, if shown to be general, can explain both the higher effectiveness of authentic uronium salts and short preactivation times.^[23, 24]

A particularly clear indication of the enhanced reactivity of the O-series has been noted during a study of peptide cyclization (Table 2). On treatment with N-HBTU, more than 91% of the linear peptide is still unreacted after 15 s, whereas with O-HBTU over half has disappeared. A slightly greater yield of the *all*-L cyclic monomer is obtained with N-HBTU than with the O-isomer, and in contrast to segment condensation, the O-form results in almost twice the amount of epimerization. The reasons for the difference are not clear, although such effects have been observed in other cases between cyclization and ordinary segment condensation^[25]

Table 2. Cyclization of H-Ala-Ala-Ala-BnAla-Ala-Ala-OH with O- and N-isomers of HBTU and HATU, %^[a]

	O-HBTU	N-HBTU	O-HATU	N-HATU
<i>all</i> -L-linear peptide ^[b]	11.6(48.4) ^[c]	9.0(91.2) ^[c]	7.5(27.7) ^[c]	0(45) ^[c]
<i>all</i> -L-cyclic monomer	31.4(18.4)	37.6(3.0)	56.3(46.5)	68.4(32.0)
epimeric cyclic monomer	7.5	4.2	4.1	< 4

[a] 10⁻³ M in DMF, coupling reagent (1.1 equiv), TMP (2 equiv), DIEA (1 equiv), HPLC analysis after 60 min. [b] epimerized linear precursor cannot be excluded. [c] values in parentheses are HPLC values after 15 sec.

and may reflect subtle variations in the extent of oxazolone formation, which could precede the two types of reaction.

Supporting Information Available: Experimental details for the synthesis of O-HBTU and O-HATU as well as appropriate IR and NMR spectra and the results of comparisons between the (a) O- and N-forms and (b) low and extended preactivation in model syntheses.

Abbreviations

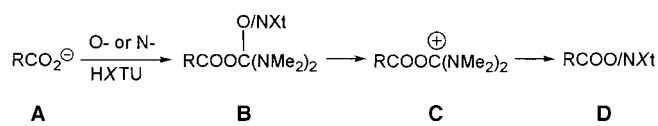
Aib: α -aminoisobutyric acid, **ACP**: acyl carrier protein decapeptide, **BOP**: benzotriazol-1-yl-*N*-oxy-tris(dimethylamino)phosphonium hexafluorophosphate, **DIEA**: *N,N*-diisopropylethylamine, **HAPyU**: 1-(1-pyrrolidinyl-1*H*-1,2,3-triazolo[4,5-*b*]pyridinylmethylene)pyrrolidinium hexafluorophosphate 3-oxide, **N-HATU**: 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium hexafluorophosphate 3-oxide, **O-HATU**: *N*-[(1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yloxy)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate, **N-HBTU**: 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide, **O-HBTU**: *N*-[(1*H*-benzotriazol-1-yloxy)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate, **HOAt**: 7-aza-1-hydroxybenzotriazole, **HOBt**: 1-hydroxybenzotriazole, **PyBOP**: benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate, **TBTU**: tetrafluoroborate analogue of HBTU, **TMP**: 2,4,6-trimethylpyridine (collidine), **Z**: benzyloxycarbonyl.

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based on $|F|$, and by using anisotropic displacement parameters for P, O, F, C, and N atoms with fixed H atoms (CRYSTALS 2000: D. J. Watkin, C. K. Prout, J. R. Carruthers, P. W. Betteridge, R. I. Cooper, Chemical Crystallography Laboratory, University of Oxford, Oxford, UK). Crystal structure data for **1b** ($\text{C}_{10}\text{H}_{15}\text{OF}_6\text{N}_6\text{P}$) at 294 K: monoclinic, space group $P2_1/n$, $a = 6.3787(6)$, $b = 19.696(5)$, $c = 13.330(3) \text{ \AA}$, $\beta = 99.94(1)^\circ$; $V = 1649.6(6) \text{ \AA}^3$; $Z = 4$; $\rho_{\text{calcd}} = 1.531 \text{ g cm}^{-3}$; crystal dimensions $0.18 \times 0.18 \times 0.72 \text{ mm}$; $R = 0.0630$; $R_w = 0.0912$, based on 1923 observed reflections ($I > 1.96 \sigma(I)$); 3044 total; $2\theta_{\text{max}} = 67^\circ$) and 218 refined parameters, including isotropic extinction correction. Decay and absorption corrections (transmission factors 0.64–0.67) were applied to the data; maximum residual electron density 0.46 e \AA^{-3} . Crystal structure data for **1a** ($\text{C}_{11}\text{H}_{16}\text{OF}_6\text{N}_6\text{P}$) at 294 K: monoclinic, space group $P2_1/c$, $a = 8.3999(13)$, $b = 28.799(4)$, $c = 7.0528(5) \text{ \AA}$, $\beta = 109.857(9)^\circ$; $V = 1604.7(4) \text{ \AA}^3$; $Z = 4$; $\rho_{\text{calcd}} = 1.570 \text{ g cm}^{-3}$; crystal dimensions $0.29 \times 0.58 \times 0.58 \text{ mm}$; $R = 0.0614$; $R_w = 0.0786$; based on 2530 observed reflections ($I > 1.96 \sigma(I)$); 3440 total; $2\theta_{\text{max}} = 78^\circ$) and 272 refined parameters, including description of 50:50 disorder of the PF_6^- anion and isotropic extinction correction; maximum residual electron density 0.32 e \AA^{-3} . CCDC-163597 and CCDC-163598 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB21EZ, UK; fax: (+44) 1223-336-033; or deposit @ccdc.cam.ac.uk).

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 [24] The exact nature of the intermediates formed in a particular case depends on the substrate, solvent, identity and amount of base, and presence or absence of HOXt. For example, with N-TBTU in CH_2Cl_2 in the presence of 2 equivalents of DIEA, Z-Gly-Ala-OH is converted into oxazolone in less than 2 min and OBt ester is formed only slowly ($> 1 \text{ hr}$). If only 1 eq of DIEA is present or if 1 eq of HOBt is added, complete OBt ester formation occurs within 30 sec and the ratio does not change much thereafter. In the case of Fmoc-Aib-OH, N-HATU activation shows oxazolone ($1690, 1829 \text{ cm}^{-1}$) along with O- (1819 cm^{-1}) and N-acyl (1722 cm^{-1}) species. After 5–6 min, oxazolone disappears, and some of the O-acyl derivative undergoes conversion into the N-acyl isomer so that the 3N form becomes the predominant species. Loss of configuration during segment coupling is thought to be related to the extent of oxazolone formation if a base is present. Other labile intermediates may precede formation of the OXt ester. A possible initial intermediate, following formation of the tetrahedral intermediate **B**, is the O-acyluronium cation **C**. For a



number of hindered acids (mesitoic, pivalic) authentic *O*-acyluronium salts have been isolated and identified. For the mesitoic acid derivative this species is more reactive than the OAt ester. Thus the exact timing of the possible formation of **C** and its conversion into active ester or its direct involvement in a reaction process may help to explain differences between N-HATU and N-HAPyU or between H-HATU and N-HBTU. The lifetime of intermediates such as **C** in the presence of good nucleophiles such as OXt⁽⁻⁾ is believed to be low and their definite involvement in the activation process of any particular case has not yet been established. Intermediate **C** is most likely involved under conditions of no or low preactivation; these conditions are generally more favorable for both peptide assembly and low loss of configuration during segment coupling.

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Capillary Electrophoresis Analysis of DNA Adducts as Biomarkers for Carcinogenesis**

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Formation of DNA adducts through the formation of covalent DNA modifications by exogenous or endogenous reactive chemical agents appears to be one of the earliest events in the development of cancer. The formation of DNA adducts thus represents a detectable and critical step in carcinogenesis and thus may serve as an early biomarker.

To establish the pathological significance of DNA adducts in human diseases such as cancer, it is necessary to find high-sample-throughput methods for the simultaneous detection of the various classes of DNA adducts. For example, in the European Prospective Investigation into Cancer and Nutrition (EPIC) study, 470 000 people from nine European countries were interviewed about their dietary habits and lifestyles.^[1] Blood samples were also taken for future analysis of biomarkers, such as DNA adducts.^[2] In such studies the use of the ultrasensitive ³²P-postlabeling method^[3] has a number of drawbacks: such as the lack of automation, the use of a strong β emitter, and the fact that the simultaneous detection of DNA adducts derived from different classes of carcinogens is not possible. Approximately 300 μ g of DNA or more are

necessary for the determination of DNA adducts by liquid chromatography coupled to mass spectrometry (LC-MS).^[4] Although this method is very useful for characterizing unknown DNA adducts it cannot be used for routine analysis. The biggest problem is getting enough DNA for studies, because of the expensive hydrolysis of such large amounts of DNA, and the fact that only similar DNA adducts can be analyzed in a single analytical run. A new analytical method with a high-sample-throughput is therefore necessary to determine several classes of DNA adducts simultaneously in only 10 μ g of DNA. Fluorescence derivatization of nucleotides for the analysis of DNA adducts has been used by the research groups of Sharma and Giese since 1988^[5] to replace the ³²P-postlabeling method. However, neither the described conjugation of ethylenediamine at the 5'-phosphate group of nucleotides^[5a] followed by the derivatization with dansyl chloride or fluorescein isothiocyanate,^[5b] nor fluorescence derivatization in one step with a histidine-binding group^[5c, d] fulfills the requirements for high-sample-throughput analysis.


We present here a new method to determine DNA adducts. It involves the hydrolysis of DNA, fluorescence labeling of modified and unmodified nucleotides, micellar electrokinetic chromatography, and laser-induced-fluorescence detection (CE-LIF) with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diazas-indacene-3-propionylethylenediamine (BODIPY FL EDA) as the fluorescence marker. Selective conjugation of 2'-deoxynucleoside-3'-monophosphates (dNMPs) through the phosphate moiety to the amino linker of BODIPY FL EDA was conducted in the presence of the water-soluble carbodiimide 1-ethyl-3-(3'-*N,N'*-dimethylaminopropyl)-carbodiimide (EDC) to activate the phosphate moiety of the dNMPs.^[6] However, this approach to derivatization excludes the use of fluorescence markers or buffer systems with carboxylic acid, primary amino, or phosphate groups. The only suitable buffer system for the derivatization was *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethane sulfonic acid (HEPES) at pH 6.5. The enzymatic hydrolysis of DNA was also carried out in HEPES buffer at pH 6.0 to avoid a purification step. Comparison with ³²P-postlabeling analysis showed that digestion of calf-thymus DNA (CT-DNA) in HEPES buffer resulted in the same amounts of dNMPs as with the routinely used procedure in sodium succinate.^[7] The derivatization reaction was monitored by spectroscopic characterization (ESI-MS, ¹H, ¹³C, ¹¹B, and ¹⁹F NMR spectroscopy) of the conjugate formed between 2'-deoxyadenosine-3'-monophosphate and BODIPY FL EDA.

So far we have explored the application of this CE-LIF method to the detection of apurinic (AP) sites and to the determination of a number of endogenous and exogenous DNA adducts (Table 1) in various samples such as oligonucleotides, CT-DNA, or human DNA. The same conditions for derivatization and separation were used in all analyses. CE-LIF analysis of etheno-dAMP- (Figure 1 A) and 5-Me-dCMP-modified (Figure 1 B) oligonucleotides gave excellent separation of the dAMP and dCMP adducts from the four unmodified nucleotides. Etheno-DNA adducts are generated by the reaction of DNA bases with lipid peroxidation products derived from endogenous sources or from exposure to xenobiotics.^[8] The exact quantitation of 5-Me-dCMP in

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