**Creatine phosphate** was synthesized on a 50-mmol scale by using soluble enzymes in a batch reactor. Six liters of doubly distilled water containing 1.0 mM ATP, 1.0 mM $MgCl_2$, 10 mM mercaptoethanol, 16 mM creatine (100 mmol), 14000 U of acetate kinase, and 1100 U of creatine kinase (E.C. 2.7.3.2 from rabbit muscle) was adjusted to pH 9.0, cooled to 15 °C, and kept under argon. An aqueous solution of methoxycarbonyl phosphate ($\sim$1 M) prepared as above (115 mmol total) was added over a period of 12 h while the pH was maintained at 9.0 by addition of solid K_2CO_3 ($\sim$10 g). Enzymatic assay indicated that 55 mmol of creatine phosphate had been formed (55% based on creatine, 48% based on methoxycarbonyl phosphate). The solution was filtered through an ultrafiltration membrane (Amicon YM 10) to recover enzymes (AK >90%, CK 70%) and concentrated under vacuum to a volume of 1.0 L. Creatine phosphate was isolated as described previously[21] by selective precipitation as the barium salt and recrystallized once as a powder from H_2O/ethanol. The isolated yield of creatine phosphate was 40 mmol (15 g having >90% purity).

Calculation of Phosphorylation Potential as a Function of pH. The state of ionization for reactants and products of hydrolysis was calculated at the pH where the phosphoryl donor potential was measured. The increase (or decrease) in free energy from the measured value due to changes in ionization as the pH varies is calculated based on known pK_a values and is represented by the lines in Figure 4. The line for ATP was taken from the literature[24,32] and represents excess magnesium ion conditions. Magnesium ion effects have been ignored when calculating all other lines. The calculated line for acetyl phosphate in Figure

4 is qualitatively consistent with experimental results for the acetyl phosphate-ATP equilibrium (eq 10), under excess magnesium

$$\text{acetyl phosphate} + \text{ADP} \xrightleftharpoons{\text{AK, Mg}^{2+}} \text{ATP+ acetate} \quad (10)$$
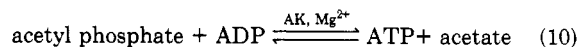
conditions.[25] Since all experimental points are based on equilibrium measurements with ATP, the absolute values of $\Delta G°_{hyd}$ are subject to uncertainties in the $\Delta G°_{hyd}$ value for ATP. The relative positions, however, will be unchanged. The pK_a values used in calculation are[33] H_3PO_4, $pK_{a2} = 7.21$; acetyl phosphate, $pK_{a2} = 4.95^{11}$; acetic acid, $pK_a = 4.76$; MCP, $pK_{a2} = 4.4$; H_2CO_3, $pK_{a1} = 6.35$ (apparent), $pK_{a2} = 10.33$; PEP, $pK_{a3} = 6.38$.

Registry No. ADP, 58-64-0; ATP, 56-65-5; AcPP, 94843-88-6; AcPPAc, 94859-21-9; MCP, 94843-85-3; MCP·2Li, 94843-86-4; PEP, 138-08-9; $H_2O_3PSSPO_3H_2$, 13598-72-6; $CH_3C(O)SPO_3H_2$, 94843-84-2; $NH_2C(O)OPO_3H_2$, 590-55-6; $CH_3CH_2C(O)OPO_3H_2$, 6659-27-4; H_3O_4P, 7664-38-2; HO_4P^{2-}, 14066-19-4; $CH_3C(O)OC-(O)CH_3$, 108-24-7; $CH_3C(O)OPO_3H_2$, 590-54-5; $CH_3CH_2C(O)OC-(O)CH_2CH_3$, 123-62-6; $CH_3OC(O)Cl$, 79-22-1; $CH_3OC(O)OC(O)-CH_3$, 94843-87-5; CH_3CO_2H, 64-19-7; H_3PO_3S, 13598-51-1; $H_2-O_3POPO_3H_2$, 2466-09-3; H_3O_3P, 13598-36-2; H_3O_2P, 6303-21-5; acetate kinase, 9027-42-3; carbamate kinase, 9026-69-1; creatine, 57-00-1; creatine phosphate, 67-07-2; pyruvic acid, 127-17-3.

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Enzymatic Synthesis of DeoxyATP Using DNA as Starting Material[1]

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We describe the synthesis of deoxyadenosine triphosphate (dATP) from DNA on a 200-mmol scale. Enzymatic digestion of DNA to a mixture of deoxynucleoside monophosphates was accomplished by a two-step process: initial conversion to a mixture of oligonucleotides using soluble DNase I, and subsequent hydrolysis of this mixture to mononucleoside monophosphates using nuclease P_1 immobilized in polyacrylamide gel. The overall conversion of the deoxynucleotide moieties present in the original DNA to soluble deoxynucleoside monophosphates was 75–90%. Selective conversion of dAMP to dATP in the presence of a mixture of dAMP, dGMP, dCMP, and TMP was accomplished by enzymatic phosphorylation using PEP, a catalytic quantity of ATP, adenylate kinase, and pyruvate kinase. DeoxyATP was isolated from the reaction mixture as its barium salt in 67% yield and 60% purity. A subsequent simple purification yielded dATP with 95% purity, in 40% overall yield based on dAMP moieties present in the starting DNA.

The deoxynucleoside triphosphates (dATP, dCTP, dGTP, TMP) are obligatory intermediates in the biologically based syntheses of DNA.[3] These four substances are commercially available but expensive.[4] We were interested in developing synthetic routes to them which would make them more readily available, both for use in the synthesis of DNA and as starting materials for other

types of syntheses. This manuscript describes work directed toward the synthesis of dATP. This compound was chosen as the target for our initial synthetic work because the enzymes required for its preparation were commercially available, because the synthetic strategy followed that of our earlier synthesis of ATP from RNA,[5-7] and because it has other interesting biological properties.[8] DeoxyATP has been synthesized previously by using enzymatic pro-

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(2) Postdoctoral Fellow of the NATO/DAAD, 1983–1984.
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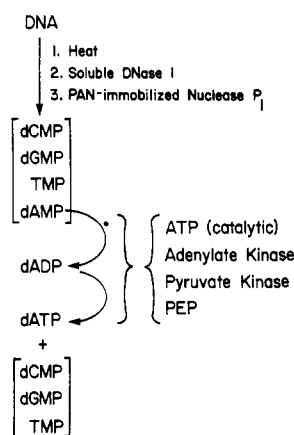
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Scheme I. Preparation of dATP from DNA

cedures but only in quantities less than 1 mmol.[7]

We used commercially available DNA as our starting material.[10] Conversion of DNA to a mixture of deoxynucleotide monophosphates was accomplished in three steps (Scheme I). The DNA was first denatured by heating. It was then treated with soluble DNase I to generate low molecular weight oligomers.[11] Finally, the solution was treated with immobilized nuclease P_1 to generate the desired mixture of deoxynucleoside monophosphates. The conversion of heat-denatured DNA to deoxynucleoside monophosphates could also be accomplished in one step by using soluble nuclease P_1. We preferred the two-stage system, however, because it permitted the use and recovery of the (expensive) nuclease P_1. (High molecular weight DNA is not a substrate for gel-immobilized nuclease P_1.) Analysis of the reaction mixture, either enzymatically or by HPLC, indicated that the conversion of dAMP moieties in the starting DNA to soluble dAMP proceeded in 93% yield.

The reaction mixture resulting from this initial set of transformations was a viscous solution containing, among other compounds, undigested oligonucleotide fragments. These polymeric species interfered with subsequent steps and were removed by treatment of the reaction mixture with 1.5–2.0 volumes of ethanol. After removal of the precipitated high molecular weight substances, the resulting clear yellow solution could be concentrated and used directly in the subsequent phosphorylation steps.

DeoxyAMP was selectively converted to dATP in the presence of the other deoxynucleotide monophosphates by enzymatic phosphorylation using a mixture of adenylate kinase, pyruvate kinase, ATP, and phosphoenol pyruvate. This selective transformation is possible because of the narrow substrate specificity of adenylate kinase: this enzyme accepts only AMP, ADP, ATP, and the corresponding 2′-deoxy species as substrates. The detailed course of the conversion of dADP to dATP is unclear. DeoxyADP is a substrate for both adenylate kinase and pyruvate kinase, and both enzymes are present in sufficient quantity to contribute to the conversion.
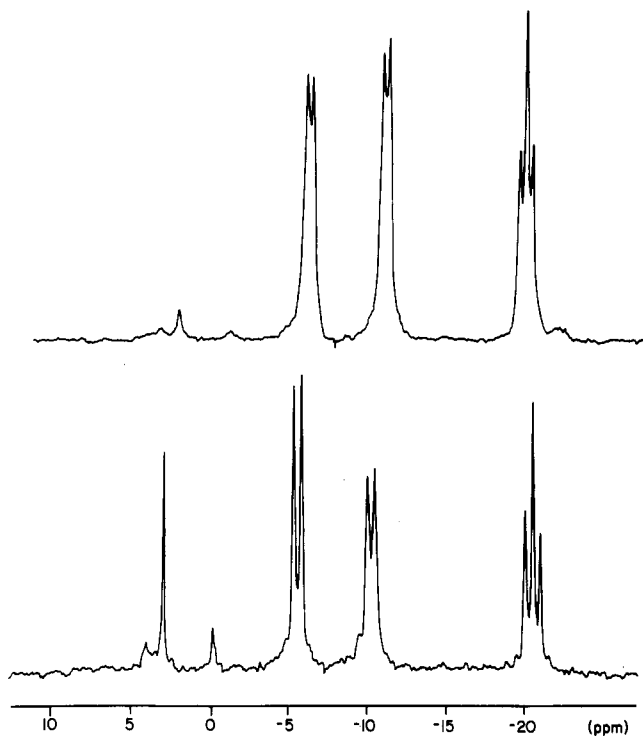

Figure 1. ^{31}P NMR spectra of crude (lower) dATP and purified (upper) dATP (c 50–75 mM, 3.2 M $(NH_4)_2SO_4$, pH 7.8, 100 mM in $MgCl_2$).

The dATP produced was isolated as its barium salt by selective precipitation. The principal impurity in the initial crude isolate was inorganic phosphate (18% by weight, determined by ^{31}P NMR spectroscopy; other phosphorous-containing impurities were present in quantities ≤5% of that of the dATP). Further purification was accomplished by redissolving the initially formed barium dATP by using an acidic ion exchange resin and then reprecipitating the dATP as its barium salt. The purity of the final product was 90–95%, and the overall conversion of deoxyadenosine moieties in the original DNA to dATP was 40% (Figure 1).

This final purification step deserves brief comment. In slightly acidic solution (pH ~5) the solubility of the barium salts of dAMP and dADP are sufficiently greater than that of dATP that they will remain in solution while the dATP precipitates. The stability of dATP under these conditions is, however, limited. It is, therefore, essential to conduct this final precipitation rapidly and to keep the reaction mixture cold. The quantity of ethanol added and the rate at which it is added are also important variables: too rapid addition, or addition of too much ethanol, results in coprecipitation of dADP with the dATP.

Experimental Section

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(10) Herring sperm DNA was obtained from Chemalog (Chemical Dynamics Corporation). This DNA costs $41.00 per mol of contained deoxynucleoside monophosphate, assuming an average molecular weight of 327 g/mol.

(11) Deoxyribonuclease I from bovine pancreas was used. This enzyme is an endonuclease. It hydrolyzes highly polymerized native or denatured DNA with the formation of a complex mixture of oligonucleotides.

Table I. Conversion of DNA to dATP

step	yield of dAXP,[a] %
1. conversion	93
2. removal of oligonucleotides by EtOH precipitation	77
3. conversion of dAMP to dATP	54
4. initial isolation of dATP	52
5. purification of dATP	40

[a] Yields are based on dAMP moieties present in the original DNA.

with 5 mM tetrabutylammonium phosphate in acetonitrile/water (1:4, pH 7.6) at a flow rate of 2.0 mL per min.

Enzyme Assays. *Soluble* adenylate kinase and pyruvate kinase were assayed as described.[12] The assays of the *immobilized* enzymes[13] and of PEP and pyruvate[14] were performed as described. The adenylate kinase assay was used to quantitate dAMP.[12]

Determination of the Activity of Nuclease P₁. The activity of nuclease P_1 was determined by measuring the concentration of adenosine, produced from the hydrolysis of 3'-AMP at 46 °C as function of time.

Sodium acetate buffer (2.0 mL, 0.1 M, pH 5.4, 0.1 M $ZnSO_4$) and 3'-AMP (0.5 mL of a 20 mM solution) were mixed and brought to 46 °C. An aliquot (0.1 mL) of a dilute solution of nuclease P_1 was added with mixing. The reaction was stirred continuously during the assay of immobilized enzyme. At intervals, aliquots (0.1 mL) of the reaction mixture were removed, and the concentration of adenosine was determined by enzymatic assay.[10]

Enzyme Immobilization. PAN (poly(acrylamide-*co-N*-(acryloxy)succinimide)) was prepared according to the literature procedure.[13] Adenylate kinase and pyruvate kinase were immobilized following literature procedures.[14] The immobilization yields were 13% and 33%, respectively.

Immobilization of Nuclease P₁. Nuclease P_1 was immobilized according to the general procedure described elsewhere.[13] The following amounts of solutions, chemicals, and enzyme were used: PAN 1000 (3.0 g) dissolved in MOPS buffer (8 mL, 0.2 M, pH 7.0); DTT (0.14 mL, 0.5 M), TET (1.6 mL, 0.5 M); nuclease P_1 (5 mg) dissolved in $ZnSO_4$ solution (2 mL, 1 mM). Because the polymerization of PAN 1000 is fast under these conditions, the enzyme solution was added *before* the addition of the TET solution. The immobilization yield was 40%.

Digestion of DNA. DNA (327 g, ~0.82 mol of deoxynucleotide moieties) was suspended in water (1.3 L). The DNA was dissolved by heating and by the slow addition (~1 h) of a 10 M KOH solution so as to maintain the pH ≤7.5. The reaction mixture was maintained at 100 °C for 20 min and then cooled in an ice bath to a temperature of 40 °C. The mixture was transferred to a constant-temperature bath at 40 °C and the enzymatic digestion started by the addition of $MgSO_4\cdot7H_2O$ (1.9 g, 7.7 mmol) and DNase I (38 mg).[15] During the digestion, the pH was maintained at 7.2 by the addition of a 10 M KOH solution using a pH controller. After 24 h, additional DNase I (20 mg) and $MgSO_4\cdot7H_2O$ (1.9 g) were added; these additions were repeated after 48 h. The reaction mixture was cooled to 0 °C in an ice bath and the resulting precipitate removed by centrifugation. The clear dark brown supernatant was transferred to a 5-L three-necked flask, and its pH was adjusted to 5.3 with glacial acetic acid. Water was added to bring the total volume to 4 L. The solution was warmed to 50 °C and the digestion of the mixture of oligodeoxynucleotides initiated by the addition of of nuclease P_1 (1796 U, immobilized in PAN 1000) and $ZnSO_4\cdot H_2O$ (115 mg, 0.4 mmol).[16] The pH was maintained at 5.3 by the addition of

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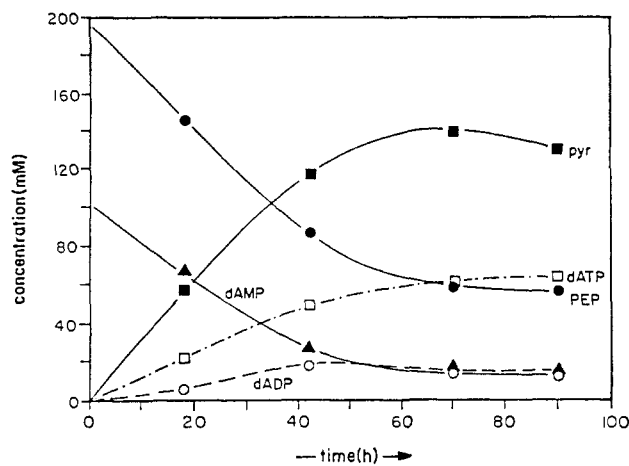
Figure 2. Course of the concentrations of dAMP, dADP, PEP, and pyruvate (Pyr) during the synthesis of dATP.

KOH solution using a pH controller. After 2 days, enzymatic assays indicated no further increase in the concentration of dAMP. The enzyme-containing gel was removed by centrifugation and washed with water (2 × 100 mL). The solution was concentrated to a volume of 1.7 L by using a rotary evaporator. Enzymatic assay indicated the presence of 190 mmol of dAMP in the solution (Table I). The concentrate was cooled to 4 °C and then diluted to 4.2 L by the addition of 95% ethanol over a period of 30 min. The resulting precipitate was removed by centrifugation, and the clear yellow supernatant was concentrated as before. The resulting residue was suspended in water (800 mL), and the insoluble material was removed by centrifugation and discarded. To the clear dark brown supernatant was slowly added 95% ethanol (1.2 L). The resulting precipitate was again removed and discarded, and the solution was concentrated. The concentrates (1050 mL) contained 158 mmol of dAMP. The activity of the immobilized nuclease P_1 recovered from this preparation was ~50% of that originally added to the reaction mixture. For storage, the enzyme-containing gel was washed with water (4 × 100 mL) and stored at 0 °C in a 1 mM $ZnSO_4$ solution. HPLC analysis of the mixture of deoxynucleotide monophosphates showed (mmol) dAMP (155), dCMP (not detected), dGMP (108), and TMP (115). Two additional peaks with retention times very similar to those of ADP and dADP were detected but were not identified.

Synthesis of DeoxyATP. A solution containing 155 mmol of dAMP in 1050 mL of solution was transferred to a 2-L three-necked flask, and the solution was warmed to 30 °C. The pH of the solution was adjusted to 7.9 by using a solution of concentrated KOH. PEP monopotassium salt (69.8 g, 325 mmol, 95% pure)[5] was added in one portion, and the pH was readjusted to 7.9. $MgSO_4\cdot7H_2O$ (762 mg, 3.8 mmol), $ATP\cdot Na_2$ (150 mg, 0.25 mmol), and DTT (100 mg) were added, and argon was bubbled through the solution for 30 min. The reaction was initiated by the addition of 322 U of adenylate kinase and 598 U of pyruvate kinase, each immobilized separately in PAN 1000. The reaction was maintained between pH 7.6 and 7.8 by addition of glacial acetic acid using a pH controller. The progress of the reaction was followed by periodic enzymatic (dAMP, PEP, pyruvate) and HPLC (dATP and dADP) assays (Figure 2). The final volume of the reactor was 1710 mL. Enzymatic assay for dATP showed a final concentration of 64 mM. The enzyme-containing gels were removed by centrifugation and washed with water (2 × 100 mL). The recovered activities of these enzymes were 90–100% of the original activities. The washes were combined with the original reaction mixture, and the resulting solution was cooled to 4 °C. A solution of $BaBr_2\cdot2H_2O$ (108 g, 325 mmol) in water was added slowly with stirring, followed by 95% ethanol (1000 mL). The solution was allowed to stir for 4 h at 4 °C, and the resulting white precipitate was collected by centrifugation. The precipitate was washed with 40% ethanol (2 × 200 mL), 95% ethanol (200 mL), and acetone (200 mL). It was dried (8 h, 25 °C, 0.05 torr) to give

(16) Nuclease P_1 requires zinc for activity: Fujimoto, M.; Kuninaka, A.; Yoshino, H. *Agric. Biol. Chem.* **1974**, *38*, 785–790.

a white solid (138 g). To determine the composition of this material, a small quantity (~10 mg) was weighed carefully and suspended in water (~5 mL). Ammonium sulfate solution (0.5 mL, 3.2 M) was added and the mixture was shaken for several minutes. The resulting solution was diluted to 10 mL, and an aliquot was analyzed by HPLC. This analysis indicated that the precipitate contained 106 mmol of dATP corresponding to a yield of 68% and a purity of 60%. Analysis of the solution from which the dATP·Ba$_2$ had been isolated indicated the following distribution of deoxynucleotide monophosphates (mmol): dAMP (6.5), dCMP (121), dGMP (9), and TMP (115).

Purification of dATP·Ba$_2$. All steps in this procedure were conducted in a cold room at 4 °C. The crude dATP·Ba$_2$ obtained as described above (20 g, 15.8 mmol of dATP) was suspended in cold water (200 mL). Ion-exchange resin (Bio-Rex 70, 100–200 mesh, Na$^+$ form, 60 g) was added and the suspension was stirred vigorously for 30 min. The resin and any undissolved material were removed by centrifugation and the separated solids were washed with water (2 × 75 mL). ^{31}P NMR spectroscopy indicated that the residual combined aqueous solutions (320 mL) contained 1 mmol of inorganic phosphate (calculated from the peak integral ratio and the assumption that all the dATP·Ba$_2$ had dissolved). After the pH of this solution had been adjusted to 4.8 with 10% aqueous HCl, BaBr$_2$ solution (2.5 mL, 0.5 M, 1.25 mmol) was added slowly with stirring. The resulting precipitate of barium phosphate was removed by centrifugation and discarded. To the clear supernatant was added BaBr$_2$ solution (50 mL, 0.5 M, 25 mmol) with stirring over 10 min. At this point, the solution had a volume of 550 mL. Ethanol (100 mL, 95%, 4 °C) was added over 10 min, and the resulting precipitate was allowed to stand for 2 h. The precipitate was collected by centrifugation and washed with 15% ethanol (150 mL), 50% ethanol (150 mL), 95% ethanol (2 × 150 mL), acetone (150 mL), and anhydrous ether (2 × 100 mL). The resulting solid was dried (0.1 torr, 25 °C, 4 h) to give 9.3 g (78% yield, 95% pure by enzyme assay) of dATP·Ba$_2$.

Polar Substituent Effects on ^{19}F Chemical Shifts of Aryl and Vinyl Fluorides: A Fluorine-19 Nuclear Magnetic Resonance Study of Some 1,1-Difluoro-2-(4-substituted-bicyclo[2.2.2]oct-1-yl)ethenes

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An extensive series of 1,1-difluoro-2-(4-substituted-bicyclo[2.2.2]oct-1-yl)ethenes (2) covering a diverse range of substituents (X = H, NO$_2$, CN, CF$_3$, COOCH$_3$, F, Cl, Br, OCH$_3$, C$_6$H$_5$, C$_2$H, C$_2$Si(CH$_3$)$_3$, CH$_3$, and C(CH$_3$)$_3$) have been synthesized and their ^{19}F NMR spectra have been recorded. Most of the compounds (except 2, X = C$_2$H) were synthesized by difluoromethylenation of the appropriate carbaldehydes which, in turn, with one exception (2, X = C$_2$Si(CH$_3$)$_3$), were all prepared from the corresponding carboxylic acids. The ^{19}F chemical shifts of 2 (δ_{F_a} and δ_{F_b}) were found to correlate very well against polar field substituent parameters (σ_F values). Most importantly, the pattern of polar susceptibility parameters ($\rho_F(F_a) > \rho_F(F_b)$) for 2 in cyclohexane was found to be similar to that previously observed for 4-substituted β,β-difluorostyrenes (1). Since the analysis of the latter system hinged critically on the dissection of polar and resonance effects by multiple linear least-squares regression analysis (DSP method), the corroboration of the conclusions drawn from 1 concerning direct field effects provides strong support for the validity of this controversial methodology.

Introduction

Although the origin of polar substituent effect contributions (ρ_F effect) to aryl ^{19}F substituent chemical shifts (SCS; sensitive monitors of π electron density perturbations at remote centers of substituted arene ring systems)[1] has been a subject of some debate in the past,[2,3] it is now generally acknowledged that two electronic transmission mechanisms are chiefly responsible, [4-7] viz., field-induced π polarization of the aromatic system (F$_{\pi \leftarrow \pi}$) and a direct electrostatic field effect acting on the π component of the C–F bond (F$_\pi$).[8] In general, the former mechanism is predominant[4,7] and, in these circumstances, polar aryl ^{19}F SCS are found to parallel the corresponding ^{13}C SCS of the unfluorinated aryl derivatives, i.e., $\Delta q_\pi{}^F \propto \Delta q_\pi{}^c$ (π electron density perturbations at the respective remote probe nuclei, viz., fluorine and carbon). However, in some situations the F$_{\pi \leftarrow \pi}$ effect is fortuitously small (e.g., 1,5 (or 5α) and 2,5 (or 5β) dispositions in 1- and 2-substituted naphthalenes, respectively)[9,10] and, as a consequence, the F$_\pi$ effect can now dominate. Here there is no longer a simple relationship between polar aryl ^{19}F and ^{13}C SCS.

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(8) In this and future publications we shall adhere to the nomenclature recently suggested by Reynolds[7] to represent various electronic substituent effects. Thus, the symbol σ_F is employed in place of σ_I in view of the overwhelming evidence that σ_I is a manifestation of polar field effects. F$_{\pi \leftarrow \pi}$ and F$_\pi$ replace F$_\pi$ and F$_D$, respectively, to represent field-induced π electron transfer between conjugated groups (e.g., fluorine and field-polarized aromatic groups) and direct π polarization (e.g., π component of the C$_{sp^2}$-F bond).

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