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The arsonomethyl analogue of adenosine 5′-phosphate

An uncoupler of adenylate kinase

Stephen R. ADAMS, Michael J. SPARKES and Henry B. F. DIXON
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

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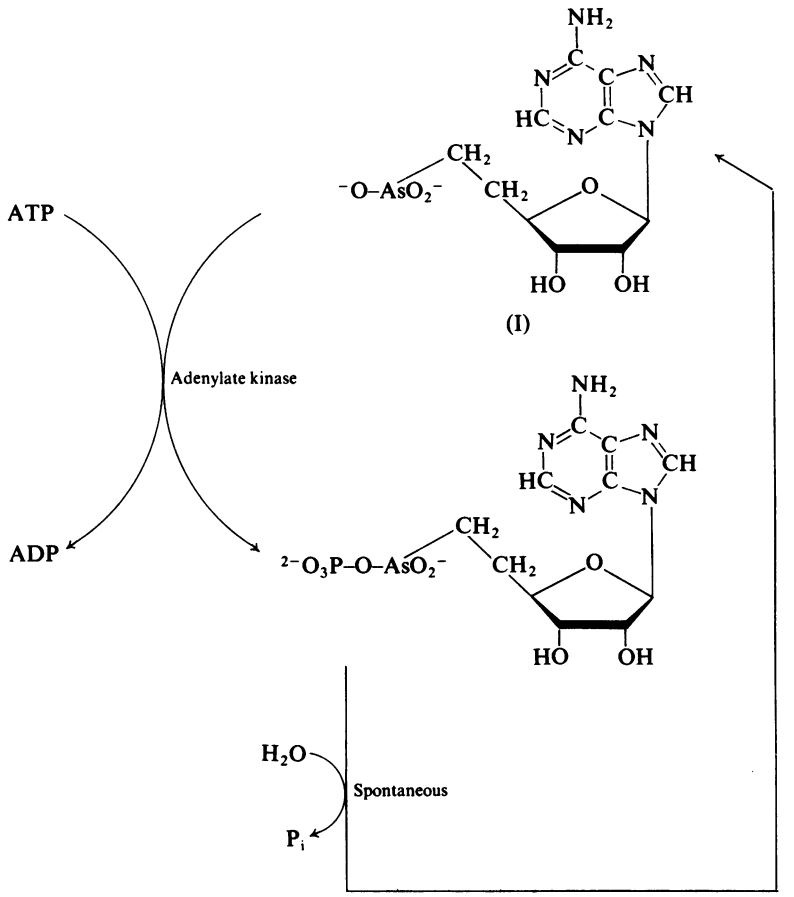
Adenosine was converted into the arsonomethyl analogue of AMP. The reactions used provide a general route for converting an alcohol, R–CH_2–OH, into the arsonomethyl analogue, R–CH_2–CH_2–AsO_3H_2, of its phosphate, R–CH_2–O–PO_3H_2. The analogue of AMP proves to be a substrate for rabbit adenylate kinase, which shows a limiting velocity with it of 1/17 that with AMP, a Michaelis constant raised 70-fold to about 10mM, and hence a specificity constant lowered about 1200-fold. The product of transfer of a phospho group from ATP to the analogue is, like all anhydrides of arsonic acids, unstable to hydrolysis, and so breaks down to yield orthophosphate and regenerate the analogue. Hence adenylate kinase is converted into an ATPase by the presence of the analogue.

The group $-AsO_3H_2$ differs from $-PO_3H_2$ only slightly in shape and charge; one of the few differences in its reactivity is that its esters are easily hydrolysed and formed. The main biochemical applications of this have involved replacement of orthophosphate by arsenate, which is incorporated into compounds that break down spontaneously (Braunstein, 1931), especially into anhydrides, such as 3-phosphoglyceroyl phosphate (Warburg & Christian, 1939) and Ado–O–*P*–O–*P*–O–AsO_3H_2 (Gresser, 1981), which are rapidly hydrolysed. Although esters of arsenic acid are also labile to hydrolysis, they are stable enough to have been shown to be substrates for many of the enzymes that act on the corresponding phosphates (Lagunas & Sols, 1968; Long & Ray, 1973; Lagunas, 1980). They may therefore be used for the same purpose as arsenate, to be incorporated into an anhydride whose hydrolysis is rapid; thus Lagunas *et al.* (1984) have shown that adenosine 5′-arsenate is a substrate for adenylate kinase (EC 2.7.4.3), so that this enzyme hydrolyses ATP in its presence, by transferring a phospho group on to the arsono group to give an ADP analogue that is rapidly hydrolysed. Nevertheless, the usefulness of esters of arsenic acid is limited by their instability, since their half-lives at neutral pH are less than 1h. Enzymes, however, often act on analogues of their substrates in which $-CH_2-PO_3H_2$ replaces $-O-PO_3H_2$ (see Engel, 1977, 1983), so it seemed possible that analogues of phosphates with both substitutions, i.e. replacement of $-O-PO_3H_2$ by $-CH_2-AsO_3H_2$, could be biochemically useful. For this reason, Webster *et al.* (1978) made such an analogue of ADP, but it proved to be a poor substrate for enzymes that act on ADP. Since, however, substitution of $-CH_2-PO_3H_2$ for $-O-PO_3H_2$ is tolerated better by enzymes that act on esters of orthophosphoric acid than by those that act on esters of diphosphoric acid, we decided to make the arsonomethyl analogue of AMP. We now report its synthesis, and that it does indeed convert adenylate kinase into an ATPase by accepting a phospho group from ATP to form an analogue of ADP that is rapidly hydrolysed to liberate orthophosphate and regenerate the AMP analogue (Scheme 1). The reactions used for its synthesis (Scheme 2) provide a route applicable also to alcohols other than adenosine for making the arsonomethyl analogues of their phosphates.

Materials and methods

General methods

Paper electrophoresis was used for assessing the products of reactions. Whatman 3MM paper was cooled by immersion in white spirit, and the following buffer solutions were used: pH2.0, 2% (v/v) formic acid/8% (v/v) acetic acid; pH3.5, 0.5% (v/v) pyridine/5% (v/v) acetic acid; pH6.5, 10% (v/v) pyridine/0.3% (v/v) acetic acid. For the pH6.5 buffer the coolant contained 8% (v/v) pyridine. A potential gradient of about 90V/cm



Scheme 1. *Action of adenylate kinase as an ATPase in the presence of the arsonomethyl analogue (I) of AMP*

was used, and phosphates, arsonates and arsenate were detected on paper by their Fe^{3+}-binding power (Wade & Morgan, 1953).

Two t.l.c. systems were used, both on cellulose (Polygram CEL 300 UV_{254} from Macherey–Nagel via Camlab, Cambridge, U.K.): system 1, ethanol/1M-ammonium acetate, pH7.5 (7:3, v/v); system 2, isobutyric acid/aq. NH_3 (sp.gr. 0.88)/water (66:1:33, by vol.). Adenine derivatives were detected by their quenching of the fluorescence of the sheets under u.v. irradiation. (A different t.l.c. system was used for characterizing isopropylideneadenosine.)

N.m.r. spectra were measured with a Hitachi Perkin–Elmer R-42 spectrometer at 60MHz.

Reagents

2',3'-Isopropylideneadenosine. Adenosine (22.4g, 84mmol) was added to a solution of 105ml of 2,2-dimethoxypropane and 210ml of acetone. The suspension was stirred, and a solution of 19.3g (102mmol) of toluene-4-sulphonic acid in 105ml of 2,2-dimethoxypropane was slowly added. After about 1h the adenosine had dissolved, and the solution was kept at room temperature (about 20°C) overnight.

The resulting solution was passed through a column (45cm × 5cm) of the strongly basic anion-exchange resin Duolite 113 SRA 70 (52–100 mesh) in the acetate form after the column had been thoroughly washed with acetone to remove all water. After the reaction mixture had passed through the column, the column was washed with its own volume of acetone. The combined effluent was evaporated to dryness. The crystalline product was suspended in acetone and filtered off. The yield was 22g (85%). T.l.c. of the product on Polygram SIL G/UV_{254} plates in ethanol/chloroform (1:4, v/v) with examination under u.v. irradiation showed that the product was free from adenosine. Recrystallization from ethanol gave fine white needles of m.p. 220–221°C [Fromageot *et al.* (1967) give 220°C].

The Wittig reagent $Ph_3P{=}CBr{-}CO{-}O{-}CMe_3$. Triphenylphosphine (52.4g, 0.2mol) and t-butyl bromoacetate (39g, 0.2mol) were dissolved in dry

toluene (250ml), and the solution was stirred overnight at room temperature. The salt $Ph_3P^+-CH_2-CO-O-CMe_3\,Br^-$, which crystallized out over this time, was filtered off, washed with dry toluene and with light petroleum (b.p. 40–60°C), and dried. The yield was 85.9g (94%), with m.p. 173°C (decomp.) [Griffiths *et al.* (1976) gave 177°C].

The salt $Ph_3P^+-CH_2-CO-O-CMe_3\,Br^-$ (22.9g, 50mmol) was dissolved in 750ml of an aqueous solution of sodium acetate (0.15M) and acetic acid (0.15M). A solution of Br_2 (8.0g, 50mmol) in water (500ml) was added dropwise with stirring, at such a rate that the solution remained colourless. The resulting solution was adjusted to pH9 with aq. NaOH (about 6M), and the product was extracted into chloroform (three 200ml portions). The solution in chloroform was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The product was recrystallized from ethyl acetate (about 350ml) on adding light petroleum (b.p. 40–60°C) (200ml) to give pale-yellow needles. The yield was 16.3g (71%), with m.p. 178°C (decomp.). N.m.r. data: δ (p.p.m.) ([^{2}H]chloroform) 1.05 [9H, s, $C(CH_3)_3$], 7.50 [15H, broad multiplet, phenyl].

Synthetic pathway (*Scheme 2*)

Step 1: conversion of the $-CH_2OH$ group into $-CHO$. The method of Pfitzner & Moffatt (1965) was used, and the details of the preparation procedure of Montgomery *et al.* (1974) were further modified as follows. 2′,3′-Isopropylideneadenosine (3.1g, 10mmol), dicyclohexylcarbodi-imide (6.2g, 30mmol) and crystalline H_3PO_4 (0.49g, 5mmol) were dissolved in 50ml of redistilled dimethyl sulphoxide, and the solution was stirred at 25°C for 4h. The dicyclohexylurea that crystallized out during this time was filtered off and washed with dimethyl sulphoxide. The resulting solution of the aldehyde was used directly in the next step.

Step 2: conversion of the $-CHO$ group into $-CH=CBr-CO-O-CMe_3$. Anhydrous pyridine (2.0ml, 25mmol) was added to the solution in dimethyl sulphoxide to neutralize the H_3PO_4, followed by 4.6g (10mmol) of $Ph_3P=CBr-CO-O-CMe_3$, and the mixture was stirred overnight at 37°C. The solution was poured into water (about 400ml), and the crude product was extracted into ethyl acetate (three 200ml portions). The solution was evaporated to dryness, and pyridine was removed by re-evaporating a few times after addition of water until no pyridine remained, which left an orange oil. Water was removed from it by evaporating a few times after addition of ethanol.

Step 3: conversion of the $-CH=CBr-CO-O-CMe_3$ group into $-CH_2-CHBr-CO-O-CMe_3$. The crude product from step 2 was dissolved in ethanol (200ml) and cooled to 0°C. $NaBH_4$ (2g, 50mmol) was added, and the solution was stirred for about 40min; a further addition of the same amount of $NaBH_4$ was then made and the stirring continued for another 40min. Water (about 800ml) was added, and the crude product was extracted into ethyl acetate (three 200ml portions). It was evaporated to dryness, and re-evaporated a few times after addition of water to remove traces of ethanol.

Step 4: conversion of the $-CH_2-CHBr-CO-O-CMe_3$ group into $-CH_2-CHBr-CO_2H$ and unblocking of O-2′ and O-3′. The oil from step 3 was dissolved in 25ml of aq. 90% (v/v) trifluoroacetic acid and kept at room temperature (about 18°C) for 2h. The solution was evaporated to dryness on a rotary evaporator with the bath at 30°C, and re-evaporated a few times after addition of diethyl ether. The product was dissolved in a mixture of water and ethyl acetate (about 200ml of each), the aqueous layer was separated, and the ethyl acetate

	Step and reagents	Isolations
$R'-CH_2OH$		
↓	1. Me_2SO, H_3PO_4, $R''-N=C=N-R''$	
$R'-CH=O$		
↓	2. $Ph_3P=CBr-CO-O-CMe_3$	
$R'-CH=CBr-CO-O-CMe_3$		
↓	3. BH_4^-	
$R'-CH_2-CHBr-CO-O-CMe_3$		
↓	4. CF_3-CO_2H	
$R-CH_2-CHBr-CO_2H$		Ion-exchange chromatography
↓	5. Alkaline arsenite	
$R-CH_2-CH(-AsO_3H_2)-CO_2H$		Ion-exchange chromatography
↓	6. Heat in propanol	
$R-CH_2-CH_2-AsO_3H_2$		Ion-exchange chromatography and crystallization

Scheme 2. *Synthetic pathway for converting an alcohol into the arsonomethyl analogue of its phosphoric ester*
In the synthesis performed, $R-CH_2OH$ was adenosine, and $R'-CH_2OH$ its 2′,3′-isopropylidene derivative.

was washed with several portions of water. The combined aqueous extracts were evaporated to dryness. The crude product was dissolved in 10ml of water and divided into two 5ml portions, each of which was worked up as follows. The portion was applied to a column (55cm × 3cm) of DEAE-cellulose (Whatman DE 52), equilibrated and eluted with a solution of pyridine (60mM) and acetic acid (150mM). Fractions were collected, and their absorbance at 290nm was read (Fig. 1*a*). The last peak to emerge (at about 3–5 column volumes) was pooled and evaporated to dryness. Residual acetic acid was removed by re-evaporating a few times after addition of water. Pyridine was removed by adjusting a solution of the product in water to pH6.5 with aq. NaOH (about 1M) and evaporating it to dryness. Repeated re-evaporation after additions of ethanol yielded a white solid. The yield was 20–25% from 2',3'-isopropylidene-adenosine, calculated on the basis of a molar absorption coefficient at 259nm for AMP of 15 400 $M^{-1} \cdot cm^{-1}$ (Bock *et al.*, 1956). The product had R_F 0.48 on t.l.c. with system 1 (see above), and a trace contaminant of R_F 0.38 was also found. Adenosine, adenine and AMP possessed R_F values of 0.60, 0.64 and 0.07 respectively in the system.

Step 5: conversion of the $-CH_2-CHBr-CO_2H$ *group into* $-CH_2-CH(-AsO_3H_2)-CO_2H$. The solid from step 4, calculated by u.v. absorbance, as above, to be 2.3mmol, was dissolved in water and added to a solution of As_2O_3 (0.59g, 3mmol) and NaOH (1.2g, 30mmol), to give a final volume of 12ml. This solution was kept at 40°C for 24h, adjusted to about pH7 with conc. HCl, diluted, and passed through a column (10cm × 2cm) of sulphonic resin (Duolite C225 SRC 15) in the H^+ form. The column was washed with water (10 column volumes), and the crude product was eluted with aq. NH_3 solution (0.5M, about 20 column volumes) until the absorbance at 290nm had fallen to negligible values. The effluent was evaporated to dryness, dissolved in water and applied to a column (55cm × 2cm) of DEAE-cellulose (Whatman DE 52), equilibrated and eluted with a solution of pyridine (120mM) and acetic acid (300mM). Fractions were collected and their absorbance at 290nm was read (Fig. 1*b*). The last peak to emerge (about 4–5 column volumes) was collected, pooled and evaporated to dryness. Residual acetic acid was removed by re-evaporating the material a few times after addition of water. A solution of the product was adjusted to pH9 with NH_3 solution and evaporated to dryness to give a yellow glass. The yield of the step was 20%, calculated as for step 4. Analysis by t.l.c. (system 1) gave a spot with R_F 0.07. The electrophoretic mobility is given in Table 1.

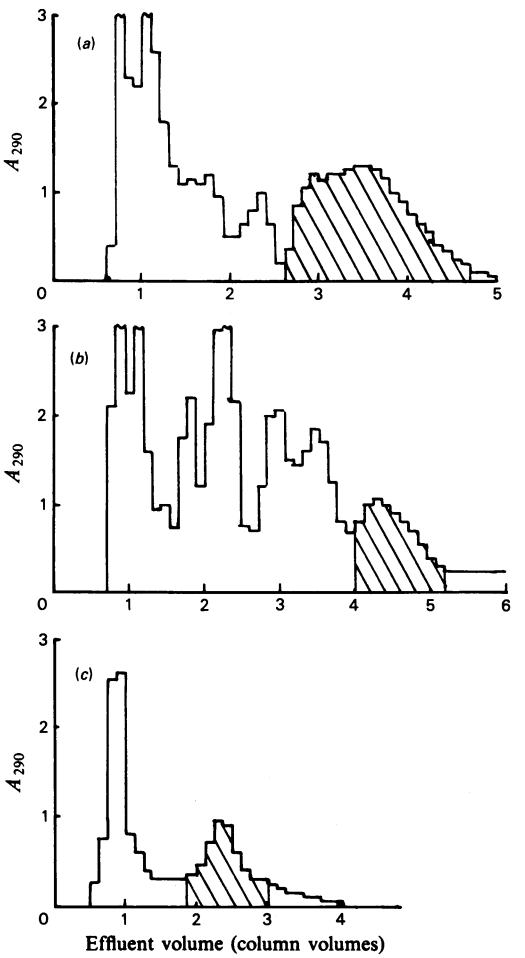


Fig. 1. *Purification of the analogue and intermediates by chromatography on DEAE-cellulose*

Preparative chromatography of: (*a*) the product from step 4 (Scheme 2), $R-CH_2-CHBr-CO_2H$; (*b*) the product from step 5, $R-CH_2-CH(-AsO_3H_2)-CO_2H$; (*c*) the product from step 6, the arsonomethyl analogue of AMP, $R-CH_2-CH_2-AsO_3H_2$ ($R-CH_2OH$ represents adenosine). Column dimensions: (*a*) 55cm × 3cm; (*b*) 55cm × 2cm; (*c*) 50cm × 2cm. Eluent: (*a* and *c*) 60mM-pyridine/150mM-acetic acid; (*b*) (from which a substance with a second acidic group, and so more strongly adsorbed, was eluted) 120mM-pyridine/300mM-acetic acid. Compounds containing adenine were detected by their absorbance at 290nm. The hatched areas represent the fractions pooled for (*a* and *b*) the next step in synthesis or for (*c*) crystallization.

Step 6: conversion of the $-CH_2-CH(-AsO_3H_2)-CO_2H$ *group into* $-CH_2-CH_2-AsO_3H_2$. An aqueous solution of the product from step 5, calculated, as above, to contain 0.46mmol, was adjusted to pH2.5 with 90% (v/v) formic acid and evaporated

to dryness. The yellow oil was dissolved in water (1–2ml), dimethyl sulphoxide (about 150ml) and propan-1-ol (about 100ml). The solution was heated to boiling, and the azeotrope between propan-1-ol and water was distilled off until the boiling point rose to that of propan-1-ol (97.2°C), when boiling was continued under reflux for 1h. The solution was cooled, evaporated to dryness in a rotary evaporator at about 80Pa (about 0.6mmHg) with the bath at 60°C, and the residue was dissolved in water (2–3ml). The solution was applied to a column (50cm × 2cm) of DEAE-cellulose (Whatman DE 52) equilibrated and eluted with a solution of pyridine (60mM) and acetic acid (150mM). Fractions were collected and their absorbances read at 290nm (Fig. 1*c*). Those representing the peak that emerged after 1.5 column volumes were pooled and evaporated to dryness. Residual acetic acid was removed by re-evaporating the material a few times after addition of water, and a white solid remained, which dissolved in water on adjustment to pH9 with aq. NH_3. The yield was 50%, calculated, as above, by absorbance at 259nm. The substance gave a single spot on t.l.c. with R_F 0.22 in system 1 and R_F 0.76 in system 2.

The free acid was obtained by passing the solution, estimated to contain 0.5mmol, through a column (6cm × 1.5cm) of a strongly basic resin (Duolite 113 SRA 70) in the acetate form, washing the column with water (5 column volumes), and eluting with acetic acid (10%, v/v) until the absorbance of the eluate at 290nm fell to a negligible value (under 20 column volumes required). The effluent was evaporated to dryness, and acetic acid was removed by re-evaporating the material a few times after addition of water. The residual solid was dissolved in aq. 90% (v/v) ethanol (100ml), and crystallized on addition of diethyl ether (35ml). The crystals were filtered off, washed with ether, and dried. The m.p. was 273–275°C. The yield was 191mg, giving an overall yield from adenosine of 2.5%, taking the M_r as 389. Elemental analysis gave: C, 33.5; H, 4.6; N, 17.6%; $C_{11}H_{16}AsN_5O_6$ requires C, 33.9; H, 4.2; N, 18.0%.

The p.m.r. spectrum of the free acid in ^2H_2O was assigned as follows: δ (p.p.m.) 2.25 [2H, multiplet, CH–CH_2–CH_2–AsO_3H_2, i.e. H-5'], 2.65 [2H, multiplet, –CH_2–CH_2–AsO_3H_2], 4.15 [2H, multiplet, H-2', H-3'], 4.70 [1H, multiplet, H-4'], 5.90 (1H, d, $J_{H\text{-}1',H\text{-}2'}$, 6Hz, H-1'], 8.20 [1H, s, H-8], 8.25 [1H, s, H-2].

Enzyme studies

Adenylate kinase incubation. Rabbit muscle adenylate kinase (10μl of a solution of 2mg/ml), obtained from Boehringer Corp., London W.5., U.K., was added to 0.50ml of a solution containing 100mM-Tris/HCl buffer, pH8.5, 10mM-$MgSO_4$, 100mM-KCl, 10mM-Na_2ATP (Boehringer Corp.) and either 10mM-Na_2AMP (Sigma Chemical Co., Poole, Dorset, U.K.) or 10mM of the arsonomethyl analogue of AMP, at room temperature (about 18°C). Samples were taken for immediate electrophoresis at pH6.5 (see Table 1) and for t.l.c. (system 2) before the enzyme was added and at various intervals afterwards. The reaction with AMP was followed by the appearance of ADP (t.l.c.) and that with the analogue by the appearance of ADP (t.l.c.) and orthophosphate (electrophoresis). In the t.l.c. system, ATP, ADP and AMP had R_F values 0.42, 0.55 and 0.66 respectively.

Adenylate kinase assay. The reactions of AMP and its arsonomethyl analogue with rabbit muscle adenylate kinase were followed in a coupled assay, in which the reaction of pyruvate kinase was limited by the ADP formation by the adenylate kinase, and the reaction of lactate dehydrogenase was, in turn, limited by the formation of pyruvate by the pyruvate kinase. The decrease in A_{340} due to oxidation of NADH by the lactate dehydrogenase was monitored, and rates were calculated on the basis of an absorption coefficient of $6220M^{-1}\cdot cm^{-1}$ for NADH (Horecker & Kornberg, 1948). Final concentrations in the cuvette (total volume 0.5ml) were: 100mM-Tris/HCl buffer, pH8.5, 100mM-KCl, 1mM-$MgSO_4$, 1mM-Na_2ATP, 1mM-phosphoenolpyruvate (the salt with 1mol.prop. of cyclohexylamine; Clark & Kirby, 1966), 0.14mM-NADH (disodium salt; Boehringer Corp.), 0.27μg or 1μg of adenylate kinase, 20 or 25μg of rabbit pyruvate kinase (EC 2.7.1.40) (Boehringer Corp.), and 12.5μg of lactate dehydrogenase (EC 1.1.1.27) (Boehringer Corp.). The enzymes were added as suspensions in $(NH_4)_2SO_4$ solution. Rates were recorded with Na_2AMP (0.05–2mM) and with its arsonomethyl analogue (the free acid, neutralized with an equimolar amount of NaOH, 2–25mM).

Results

Characterization of the product

The arsonomethyl analogue was satisfactorily characterized by its content of C, H and N; its p.m.r. spectrum agreed with that expected for the desired product. The u.v. spectrum of a 50μM solution (by weight) of the free acid neutralized with an equimolar amount of NaOH was very similar to that of 50μM commercial Na_2AMP, giving an absorption coefficient at 259nm of $14600M^{-1}\cdot cm^{-1}$, with experimental error of the value for AMP of $15400M^{-1}\cdot cm^{-1}$ (Bock *et al.*, 1956).

Table 1. *Electrophoretic mobilities of the arsonomethyl analogue of AMP and related compounds*
Mobilities are measured from the position of glucose (taken as zero to correct for electro-osmosis).

Compound	Mobility pH2.0	pH3.5	pH6.5
Arsonomethyl analogue of AMP, R-CH_2-CH_2-AsO_3H_2*	+1.5	+0.13	−0.47
AMP, R-CH_2-O-PO_3H_2	−0.05	−0.28	−0.70
ADP, R-CH_2-O-PO(OH)-O-PO_3H_2	−0.85	−0.98	−1.10
P_i	−1.5	−1.9	−1.6
Arsenate	−1.1	−1.8	−1.6
Product of step 5 (Scheme 2), R-CH_2-CH(-AsO_3H_2)-CO_2H	+0.80	−0.23	−0.98
ATP	[−1]	[−1]	[−1]

* R is such that R-CH_2OH represents adenosine.

Table 2. *Comparison of AMP with its arsonomethyl analogue as substrates for adenylate kinase*
Measurements were made at pH8.5 in 1mM-ATP. Reproducibility suggested that the values shown have probable errors of less than 20% for the analogue, and less than 10% for AMP. The catalytic constants were derived from specific limiting rates by using a value of 21kg/mol for the molar mass of the enzyme (Callaghan, 1957).

Substrate	Michaelis constant K_m (mM)	Catalytic constant k_0 (s^{-1})	Specificity constant k_0/K_m ($M^{-1}\cdot s^{-1}$)
AMP, R-CH_2-O-PO_3H_2	0.15	17	115000
Arsonomethyl analogue of AMP, R-CH_2-CH_2-AsO_3H_2	10	1	100

The electrophoretic characteristics of the product are given in Table 1. The mobilities were consistent with those expected, in view of a lower pK of about 4 typical of aliphatic arsonic acids. Thus at pH2 there should be one full positive charge on the adenine, at pH3.5 nearly a full positive charge on the adenine and some negative charge on the arsonic acid, and at pH6.5 one full negative charge on the arsonic acid.

Substrate properties of the arsonomethyl analogue of AMP

On incubation of 10mM analogue with adenylate kinase and 10mM-ATP, the formation of ADP and orthophosphate was detected within 5h, whereas ADP formation with AMP was detected within 20min. Control solutions of ATP and enzyme gave no ADP or orthophosphate in 5 days of incubation without AMP or analogue, after which they gave ADP within 20min on addition of AMP.

In the coupled assay of adenylate kinase the analogue again proved to be a substrate for this enzyme, evoking oxidation of NADH in the presence of ATP and phosphoenolpyruvate provided that adenylate kinase, pyruvate kinase and lactate dehydrogenase were all present. The rates were proportional to the amount of adenylate kinase added, so the system was suitable for measuring kinetic characteristics. We have assumed that the ADP analogue formed (Scheme 1) is hydrolysed before it could serve as a substrate for pyruvate kinase, but we could not check this assumption. If it were untrue, then the rates with analogue could be down to a half of those reported. The kinetic characteristics found, derived from direct linear plots (Eisenthal & Cornish-Bowden, 1974), or from plots of v against v/s (Dowd & Riggs, 1965), are given in Table 2.

Discussion

Syntheses

2',3'-Isopropylideneadenosine. The method of Fromageot *et al.* (1967) for treating adenosine with 2,2-dimethoxypropane was slightly modified by Webster *et al.* (1978) by adding acetone to give a homogenous reaction mixture. We have now changed the method of removing the toluenesulphonic acid used as a catalyst by passing the reaction mixture through the acetate of an anion-exchange resin, since this is simpler than neutralizing it and then repeatedly extracting the product from the sodium toluene-4-sulphonate.

The Wittig reagent $Ph_3P{=}CBr{-}CO{-}O{-}CMe_3$. Brominated Wittig reagents such as $Ph_3P{=}CBr{-}CO{-}O{-}Me$ (Märkl, 1961, 1962) had been made by acid-catalysed bromination of phosphonium salts $Ph_3P^+{-}CH_2{-}CO{-}O{-}R$ to yield $Ph_3P^+{-}CHBr{-}CO{-}O{-}R$, which could then be converted into a Wittig reagent by raising the pH. When our synthesis required a t-butyl ester, we were unable to use strongly acid conditions for the bromination,

since these would have destroyed such an ester. Less-acidic conditions involve the risk that a second bromination would be faster than the first and would prevent the reaction being stopped at Ph_3P^+–CHBr–CO–O–CMe_3, but Märkl (1962) had successfully used such conditions both in acetic acid and in chloroform. We used an aqueous acetate buffer for bromination, and obtained a solution of the monobrominated phosphonium salt, which yielded Ph_3P=CBr–CO–O–CMe_3 when the pH was raised.

Synthetic pathway. Steps 1 and 2 (Scheme 2) of the pathway require little comment, since they are well-known reactions previously used in the synthesis of phosphonate analogues of nucleotides (Jones & Moffatt, 1968; Montgomery *et al.*, 1974), although brominated Wittig reagents were not previously used. Step 3 demanded reduction of the double bond without loss of bromine, and we investigated several reagents for this. Borohydride proved to be capable of reducing such a polarized double bond (cf. Kadin, 1966), although cyanoborohydride, known to reduce even more polarized ones (Hutchins *et al.*, 1976), did not. When the methyl ester –CH=CBr–CO–O–Me was substrate, considerable reduction occurred at the carbonyl group, so we changed to the t-butyl ester to provide steric hindrance to attack at the carbonyl group, enabling the double bond to be more specifically reduced.

After the reduction, step 4 is a deprotection both of the carboxy group and of the 2′- and 3′-hydroxy groups, and this allows the product to be isolated by ion-exchange chromatography. Reduction has introduced a chiral group, so the product of step 4 is likely to be a mixture of diastereoisomers, and this holds true for the product of step 5 as well.

Step 5 is a classical Meyer (1883) reaction. It has to be done in strong alkali for the paradoxical reason of preventing hydroxide from competing successfully with arsenite in expelling bromide; the competing reaction with hydroxide is likely to be first-order in hydroxide, whereas, if the reactive form of arsenite is AsO_3^{3-}, its concentration will be second-order in hydroxide, at least at pH values where the predominant form of arsenite is $(HO)_2As–O^-$, according to the equilibrium:

$$H_2AsO_3^- + 2OH^- \rightleftharpoons AsO_3^{3-} + 2H_2O$$

Step 6 is decarboxylation that we have found to be general for 2-arsonocarboxylic acids. It allows the carboxy group to be removed at the end of the synthesis, a step essential to the whole strategy used, since the presence of this carboxy group made possible the early stages of the synthesis. Most importantly, it made step 2 possible, because a Wittig reagent must contain a group such as –CO–O–R to be stable and easily made, especially to be insensitive to air, to water, and to weak acids such as the functional groups of adenine. The carboxy group also assisted step 5, by activating the bromine atom for replacement, and by rendering the compound that contained it soluble in water. Further, it made the product of step 4 purifiable by ion-exchange chromatography, i.e. on a cheap medium that is easily regenerated for repeated use.

The method of decarboxylation makes use of the procedure that we have used to esterify arsonic acids. They are easily converted into diesters by dissolving them in propan-1-ol and distilling off the azeotropic mixture of water and propanol that is formed, since this boils at a temperature much lower than propanol itself (Adams *et al.*, 1983). When 2-arsonocarboxylic acids (i.e. arsonoacetic acid with or without an alkyl substituent on C-2) are subjected to this procedure, they are decarboxylated, whereas heating them in water slowly breaks both C–C and C–As bonds. We think that the rapid, and easily reversed, esterification of the arsono group leaves the carboxy group as the strongest acid present, and provides enough electron attraction to decarboxylate the occasionally formed carboxylate ion. Protonation on an arsenic-bound oxygen atom may be involved, since salts do not decarboxylate as well as the free acid.

Application of the route to other alcohols may be prevented by the functional groups they contain. We protected the 2′- and 3′-hydroxy groups of adenosine for step 1, although they might be expected to react more slowly than the primary 5′-hydroxy group. When unprotected, they did not interfere with step 5, although in a flexible chain hydroxy groups at this distance from the bromine atom would displace it in alkali faster than the arsenite could do so.

Substrate properties

Table 2 establishes that the arsonomethyl analogue of AMP is a passable substrate for adenylate kinase. The analogue therefore constitutes a new type of uncoupling agent, entering reactions in which a phospho group is itself substituted, but forming an unstable product so that the substituting agent, here ATP, is used up (Scheme 1).

The similarity of $–PO_3H_2$ and $–AsO_3H_2$ groups was classically applied in biochemistry to the use of arsenate itself, and more recently to unstable esters of arsenic acid (see, e.g., Lagunas *et al.*, 1984); crystallographic studies have re-emphasized this similarity (Falvello *et al.*, 1977; Kamiya *et al.*, 1983). We had earlier shown substrate properties in an arsonomethyl analogue (Adams *et al.*, 1983), but only for the analogue of 3-phosphoglycerate, which is not a substrate for an enzyme that catalyses further substitution of its phospho group,

so this did not give the uncoupling effect now reported.

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