Synthesis of Conjugates of 11α-Hydroxyprogesterone with β-Nicotinamide Adenine Dinucleotide (β-NAD) and Adenosine Triphosphate (ATP)

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Conjugates of 11α-hydroxyprogesterone with adenosine triphosphate and nicotinamide adenine dinucleotide have been prepared in which the steroid is attached to the nucleotides *via* bridges from C-11 to the terminal phosphate and C-8 positions of ATP, and to the N-6 and C-8 positions on the adenine ring of NAD. Preliminary evaluation of the bioluminescence of these conjugates is reported.

Radioimmunoassay [1] (RIA) is a well established diagnostic and analytical procedure in the steroid field, in monitoring therapy, in detecting errors of metabolism, and in evaluating endocrine function. However, RIA poses problems associated with the use of radioactive materials: the short shelf-life of iodinated tracers, the disposal of radioactive waste, and the high cost of equipment. To this end much work has been focussed on developing alternative analytical procedures which use non-isotopically labelled steroid tracers. Procedures based upon fluorescent markers,[2,3] chemiluminescent markers,[4–7] bioluminiscent markers[4,8–10] and enzymes[11] have been developed. Fluorescence, chemiluminescence, and enzyme immunoassay have recently been reviewed.[12]

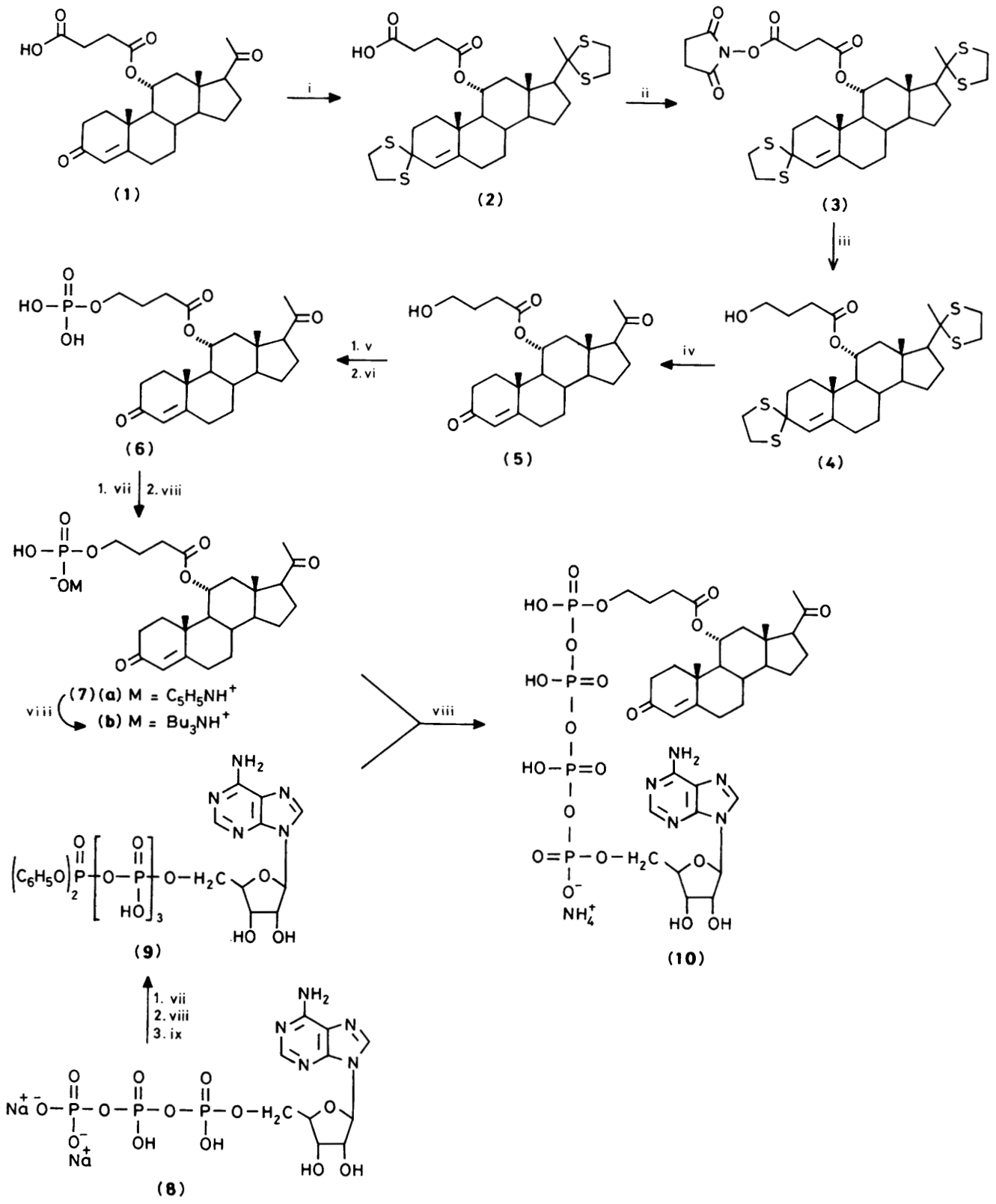
Bioluminescent markers, in particular ATP[13,14] (firefly bioluminescence), and NADH and NADPH[13,15,16] (bacterial luminescence), have received particular attention since analytical procedures based on bioluminescent reactions should be of very high sensitivity.[13] The steroid must, of course, be coupled to the co-enzyme in a position that permits the conjugate to initiate light production in the bioluminescent reaction. However, an immunoassay recently developed for progesterone using a conjugate prepared from progesterone 3-*O*-carboxymethyloxime and N^6-[(6-aminohexyl)carbamoylmethyl]–NAD found these methods to be less sensitive than comparable immunoassays.[8] This may partly be a consequence of linking the NAD derivative to progesterone *via* a bridge to the steroidal C-3 position, which is better left free for recognition by the antibody.

In this paper we present the synthesis of four conjugates of 11α-hydroxyprogesterone, (**10**), (**15**), (**16**), and (**17**), prepared from ATP (**8**), 8-(6-aminohexyl)aminoadenosine-5′-triphosphate (**12**), 8-(6-aminohexyl)aminonicotinamide adenine dinucleotide (**13**) and N^6-[(6-aminohexyl)carbamoylmethyl]-nicotinamide adenine dinucleotide (**14**) respectively in which the bridge is linked to the steroid at C-11 (Schemes 1 and 2). Experiments have shown[17] that antisera to progesterone are raised more specifically if the site of conjugation to the protein is through positions on rings B or C, usually the 11α-position, to leave the A and D rings and the side chain free to induce specificity in the antibody. It follows, therefore, that labelled tracers for recognition by an antiserum to 11α-conjugated progesterone should also have the label attached to the 11α-position.

Preparation of 11α-Hydroxyprogesterone conjugates with ATP.—Conjugates of 11α-hydroxyprogesterone and ATP have been prepared by attaching the steroid *via* a bridge to the C-8 position on the adenine ring [to give conjugate (**15**), Scheme 2] and to the terminal phosphate *via* the steroid phosphate (**7**) [to give conjugate (**10**), Scheme 1]. Our basis for the latter approach was results obtained on ligand-ATP conjugates,[18] using 2,4-dinitrobenzene as a model ligand, which showed that conjugates in which the ligand and ATP were joined *via* the phosphate chain produced higher light yields than conjugates in which the ligand was attached to the C-8 and N-6 positions of the adenine ring. During reaction with luciferin and firefly luciferase the tetraphosphate is considered[18] to break down to ATP and ligand phosphate allowing ATP and hence the original concentration of the conjugate to be determined. The 2,4-dinitrobenzene-ATP conjugates were measured quantitatively at nM concentrations.

Our route to the conjugate (**10**) is shown in Scheme 1. 11α-Hydroxyprogesterone hemisuccinate (**1**) was prepared from 11α-hydroxyprogesterone and succinic anhydride in refluxing pyridine according to a published procedure.[17] The hemisuccinate (**1**) was transformed into the primary alcohol (**5**) and then into the progesterone phosphate derivative (**6**) by the sequence shown. The oxo groups at C-3 and C-20 were protected as the bisethylene thioacetal derivative (**2**) by reaction of the hemisuccinate (**1**) with ethane-1,2-dithiol and boron trifluoride–diethyl ether in highly concentrated solutions.[19] Ethylene thioacetals of steroidal 4-en-3-ones can be prepared readily[20] but the less reactive 20-ketones require more vigorous conditions. Use is made of the remarkable solvent power of ethane-1,2-dithiol: the hemisuccinate (**1**) is allowed to react with 4 equiv. of ethane-1,2-dithiol and $BF_3{\cdot}Et_2O$ in the absence of solvent to give the 3,20-bisethylene thioacetal (**2**) in greater than 80% yield.

The use of *N*-succinimido esters to mediate peptide bond formations is a standard procedure in peptide synthesis.[21] More recent examples of the use of *N*-succinimido esters are to be found in enzyme labelling of steroids.[22,23] These derivatives offer the advantage over *in situ* methods of activation that the ester can be isolated and thoroughly characterized. *N*-Succinimido esters also offer a convenient method of reducing carboxylic acids directly to alcohols.[24] Thus treatment of the hemisuccinate (**2**) with ethyl chloroformate, to generate the mixed anhydride, and subsequent treatment, *in situ*, with *N*-hydroxysuccinimide gave the *N*-succinimido ester, (**3**), which could be purified directly from the crude product by recrystallization, in 63% yield. The ethyl ester (**18**) was the only by-product (16%), formed by rearrangement of the intermediate mixed anhydride. Reduction of the *N*-succinimido ester (**3**) with sodium borohydride in tetrahydrofuran then gave 3,3:20,20-bisethylenedithio-11α-(4-hydroxybutyryloxy)pregn-4-ene (**4**) in greater than 70% yield. Oxidative hydrolysis of the thioacetals was carried out by treatment with *N*-chlorosuccinimide and silver nitrate in aqueous acetonitrile[25] to give 11α-(4-hydroxybutyryloxy)pregn-4-ene-3,20-dione (**5**) in 52% yield. The procedure normally preferred for this reaction is to add a solution of the thioacetal to a homogeneous solution of *N*-chlorosuccinimide and silver nitrate; procedures in which the



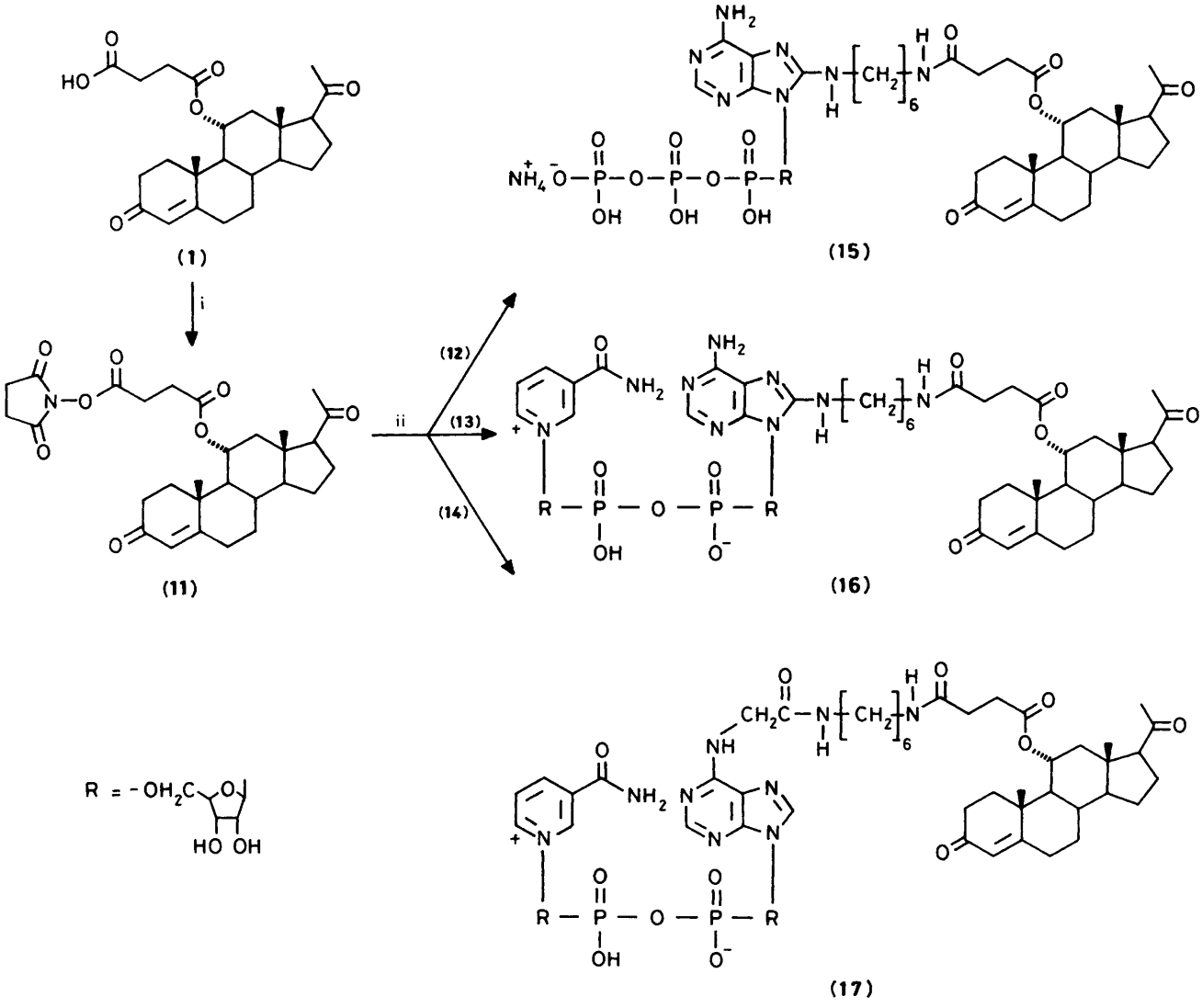
Scheme 1. *Reagents:* i, $HSCH_2CH_2SH$–BF_3·Et_2O, room temp.; ii, $ClCO_2Et$–*N*-hydroxysuccinimide, Et_3N, MeCN, room temp.; iii, $NaBH_4$, THF; iv, *N*-chlorosuccinimide–$AgNO_3$; v, $POCl_3$–Et_3N, THF, room temp.; iv, H_2O–THF, 80 °C; vii, Dowex 50W-X8, pyridinium form; viii, $Bu^t{}_3N$–DMF; ix, $(PhO)_2P(O)Cl$–DMF, room temp.

reagents are added to the thioacetal are reported to give much lower yields.[25] In our case, however, it was necessary to use the inverse method of addition because of the very low solubility of the thioacetal (**4**) in aqueous acetonitrile.

Treatment of the 3,20-dione (**5**) with phosphoryl chloride and triethylamine in tetrahydrofuran[26] gave the phosphorodichloridate (**19**). Hydrolysis with aqueous THF under reflux then gave the dihydrogen phosphate (**6**) which was purified by reversed phase h.p.l.c. [mobile phase aqueous 0.01M NaCl–acetonitrile (1:1)] followed by treatment with Dowex 50W-X8 prepared in the H^+ form. It was shown to be homogeneous by reversed phase h.p.l.c. using buffered mobile phases [aqueous 0.1M NaH_2PO_4–methanol (2:3) and aqueous 0.01M NaH_2PO_4–methanol (1:1)]. The dihydrogen phosphate (**6**) was then converted into its mono-pyridinium salt (**7a**) using a column of Dowex 50W-X8 prepared in the pyridinium form.[27]

The final step in the synthesis of the conjugate (**10**) was the condensation of the tributylammonium salt (**7b**) of the steroidal phosphate with ATP, in the form of an activated derivative. The di-sodium salt of ATP (**8**) was converted into its mono-pyridinium salt using a column of Dowex 50W-X8 prepared in the pyridinium form.[27] The aqueous solution of this salt was then treated with tributylamine for 15 min after which the residual tributylammonium salt of ATP was made anhydrous by repeated evaporation, under reduced pressure, of dry pyridine and then dry dimethylformamide. The anhydrous tributylammonium salt of ATP was then treated with diphenyl chlorophosphate in dimethylformamide for 20 h to give the activated derivative (**9**) of ATP. The steroidal mono-pyridinium salt (**7a**) was converted into the tributylammonium salt (**7b**) by treatment with an aqueous solution of tributylamine, then made anhydrous by removal of water and repeated evaporation, under reduced pressure, of dimethylformamide. The resulting anhydrous tributylammonium salt (**7b**) was then condensed with the adenosine tetraphosphate derivative (**9**) in dry dimethylformamide. The crude product was chromatographed using a column of DEAE cellulose, prepared in the HCO_3^- form to give the required ammonium trihydrogen P^1-[3-(3,20-dioxopregn-4-en-11α-yloxycarbonyl)propyl]-P^4-adenosine-5′-tetraphosphate (**10**) as a glass in 14% yield.

Other methods for activating the condensation between ATP and the tributylammonium 3-(3,20-dioxopregn-4-en-11α-yloxycarbonyl)propyl hydrogen phosphate (**7b**) were attempted. The terminal phosphate of ATP was activated as the *N*-succinimido ester and as a phosphoryl imidazolide, prepared from *N*,*N*′-carbonyldi-imidazole. A third variation was the reaction of

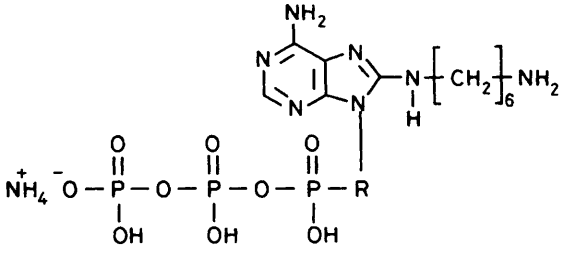


Scheme 2. *Reagents:* i, $ClCO_2Et$–*N*-hydroxysuccinimide, Et_3N, MeCN, room temp.; ii, Et_3N–DMSO, room temp.

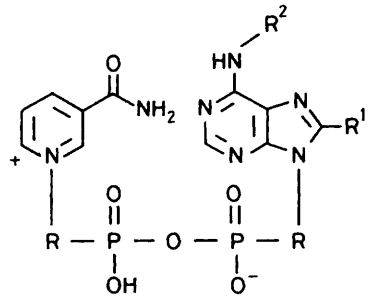
ATP, as its tributylammonium salt, with the diphenyl pyrophosphate derivative of (**5**). None of these methods, however, yielded the required conjugate (**10**).

The second conjugate of progesterone with ATP, ammonium 8-[10-(3,20-dioxopregn-4-en-11α-yloxycarbonyl)-8-oxo-7-azadecyl]aminoadenosine triphosphate (**15**), was prepared as shown in Scheme 2. 11α-[3-(Succinimido-oxycarbonyl)-propionyloxy]pregn-4-ene-3,20-dione (**11**) was prepared from 11α-hydroxyprogesterone hemisuccinate (**1**) by reaction with ethyl chloroformate followed by *N*-hydroxysuccinimide in a manner analogous to that for the *N*-succinimido ester (**3**). In this case, however, purification involved preparative h.p.l.c. prior to recrystallization. The required derivative of ATP, 8-(6-aminohexyl)aminoadenosine triphosphate (**12**) was prepared following a published procedure[27] starting from adenosine monophosphate, which involved bromination to give the 8-bromo derivative, displacement of bromine with 1,6-diaminohexane, protection of the primary amine as the trifluoroacetamide, addition of pyrophosphate and removal of the protecting group in alkali. The nucleotide (**12**) was then dried by repeated addition and evaporation, under reduced pressure of dry dimethyl sulphoxide to constant weight. A solution of the nucleotide (**12**) in dimethyl sulphoxide was then treated with the *N*-succinimido ester (**11**) in the presence of triethylamine for 6 days at room temperature. After removing dimethyl sulphoxide the crude product was extracted with dichloromethane to remove excess of organic material and the water-soluble residue was purified by preparative paper chromatography using a freshly prepared mobile phase of butan-1-ol–acetic acid–water (5:2:3, v/v). The required conjugate (**15**) was isolated in 58% yield.

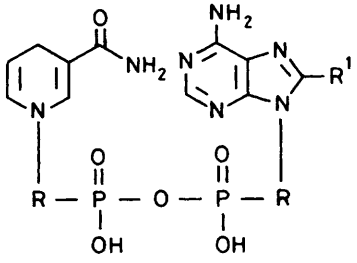
Preparation of Progesterone Conjugates with NAD.—The syntheses of the two conjugates of 11α-hydroxyprogesterone with NAD, 8-[10-(3,20-dioxopregn-4-en-11α-yloxycarbonyl)-8-oxo-7-azadecyl]aminonicotinamide adenine dinucleotide (**16**) and N^6-{[10-(3,20-dioxopregn-4-en-11α-yloxycarbonyl)-8-oxo-7-azadecyl]carbamoylmethyl}nicotinamide adenine



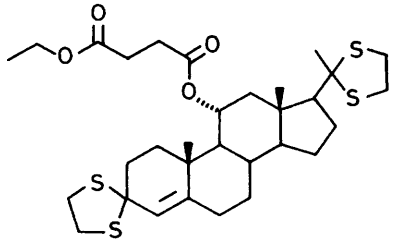
(**12**) R = -OH_2C (ribose) HO OH



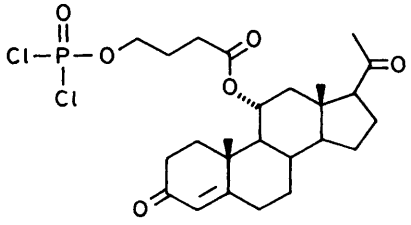
(**13**) R^1 = $NH(CH_2)_6NH_2$, R^2 = H
(**14**) R^1 = H, R^2 = CH_2-CO·$NH(CH_2)_6NH_2$
(**21**) R^1 = Br, R^2 = H



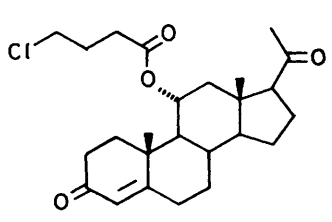
(**23**) a; R^1 = Br
b; R^1 = $NH(CH_2)_6NH_2$



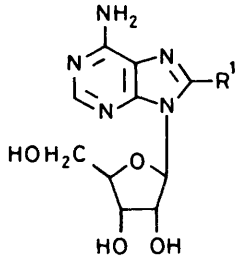
(**18**)



(**19**)



(**20**)



(**22**) a; R^1 = H
b; R^1 = Br

dinucleotide (**17**) are shown in Scheme 2. The preparation of 8-(6-aminohexyl)amino-NAD (**13**) was based upon a published procedure[28] starting from NAD. Reaction of NAD with a 13.5 molar excess of bromine in a 1.0M aqueous NaOAC–HOAc buffer at pH 4.0 gave 8-bromonicotinamide adenine dinucleotide (**21**) which was purified using a column of Dowex 1-X8 prepared in the acetate form. It is of interest to note here that whereas bromination of NAD required the use of molecular bromine, adenosine (**22a**) was converted into its 8-bromo derivative (**22b**) in greater than 70% yield using pyridinium hydrobromide perbromide.[29] Before displacement of bromine with 1,6-diaminohexane, to give the required 8-(6-aminohexyl)amino NAD (**13**), could be carried out it was necessary to protect the heat labile nicotinamide-ribose bond by reducing 8-bromo NAD (**21**) to its NADH analogue (**23a**), using yeast alcohol dehydrogenase and ethanol in a buffer solution of 1,6-diaminohexane–carbonate. The reaction was monitored by the increase in absorbance at 340 nm. Reaction of the resulting 8-bromo NADH with 1,6-diaminohexane in dimethyl sulphoxide at 60 °C for 6 h gave the 8-(6-aminohexyl)amino NADH (**23b**) which was isolated in 19% yield after chromatography using DEAE cellulose prepared in the HCO_3^- form. Oxidation of 8-(6-aminohexyl)amino NADH (**23b**) with yeast alcohol dehydrogenase and acetaldehyde in an ammonium hydrogen carbonate buffer solution then gave 8-(6-aminohexyl)amino NAD (**13**) after chromatography using DEAE cellulose prepared in the HCO_3^- form. A full description of this procedure is given in the Experimental section.

The second nucleotide, N^6-[(6-aminohexyl)carbamoylmethyl]nicotinamide adenine dinucleotide (**14**) is commercially available.

Condensation of the two nucleotides (**13**) and (**14**) with the *N*-succinimido ester (**11**) was carried out following the procedure described above for the preparation of the ATP conjugate (**15**). The yields of the two conjugates (**16**) and (**17**) were 48 and 73% respectively.

Preliminary evaluations* of these conjugates have been obtained by measuring the yield for the bioluminescent reactions over the range 1 fmol to 1 nmol. Results obtained indicate that the N^6-conjugate, N^6-{[10-(3,20-dioxopregn-4-en-11α-yloxycarbonyl)-8-oxo-7-azadecyl]carbamoylmethyl} NAD (**17**) should be investigated further to test its potential in immunoassay. The bioluminescence of this compound was 20% of that of NAD and NADH. The C-8 conjugate of NAD, (**16**), was much less efficient, bioluminiescence being *ca.* 1 000 times weaker than for NAD itself. The bioluminescent yield from the conjugates of progesterone with ATP, (**19**) and (**15**), were both *ca.* 1% of that from ATP.

Experimental

M.p.s were determined on a Reichert melting point microscope. I.r. spectra were determined for potassium bromide discs and for solutions in chloroform. N.m.r. spectra were determined at 100 MHz for solutions in deuteriochloroform with tetramethylsilane as internal standard or for solutions in [$^2H_6$]dimethyl sulphoxide with the [$^2H_5$]DMSO peak at δ 2.49 as internal reference or for solutions in deuterium oxide with HOD peak at δ 4.67 as internal reference. In lists of ^1H assignments, 'A' and 'N' refer, respectively, to protons on the adenine and nicotinamide rings. U.v. spectra were determined for solutions in water or methanol using a Unicam SP800 ultraviolet spectrophotometer. Mass spectra were determined at P.C.M.U. Harwell using a fast-atom bombardment (f.a.b.) source attached to a VG ZAB IF mass spectrometer for solutions in thioglycerol. Elemental analyses were determined by the microanalytical service, U.L.I.R.S., at University College, London. All solvents were purified before use.[30] 'Hexane' refers to the light petroleum fraction of b.p. range 60—80 °C. Dimethyl sulphoxide (DMSO) was dried over barium oxide for 24 h then over calcium oxide for 24 h followed by distillation at reduced pressure, the fraction b.p. 50—52 °C at 1.5 mmHg being collected. Dimethylformamide (DMF) was dried over magnesium sulphate and calcium sulphate for 24 h then over barium oxide for 24 h followed by distillation at reduced pressure, the fraction of b.p. 28—30 °C/1 mmHg being collected. Redistilled DMF and DMSO were stored over frame-dried 4A molecular sieve in the dark. Tributylamine was redistilled, b.p. 54—56 °C/1 mmHg and was stored in the dark over 4A molecular sieve. Pyridine was dried over sodium hydroxide under reflux, then distilled from sodium hydroxide, b.p. 114—116 °C, and was stored in the dark over 4A molecular sieve. 1,6-Diaminohexane was redistilled, b.p. 54—60 °C at 2.5 mmHg. The redistilled material had m.p. 34—38 °C (lit.,[30] 42 °C). Water was distilled from glass apparatus and then filtered through a 'Norganic' cartridge (Millipore Ltd).

Chromatographic Procedures.—Diethylaminoethyl cellulose DE-52 was prepared in the HCO_3^- form by making a slurry of Whatman DE-52 (100 g) in aqueous ammonium hydrogen carbonate (1 l of 0.5M solution), filtering under suction, and then washing the cake with water (3 litres) until the washings had constant pH. Columns were packed as slurries in water and were allowed to settle under gravity. Dowex 1-X8 was purchased in the Cl^- form from B.D.H. Chemicals Limited, Poole, Dorset. It was converted into the ^-OAc form by packing a column (100 ml) in water, washing the column with water (150 ml), and then washing it successively with aqueous NaOAc (1 700 ml of a 3.0M solution) until Cl^- ions were no longer detected in the eluate, water (150 ml), aqueous NaOAc–AcOH buffer (pH 4, 350 ml of a 1.0M solution) until the eluate had constant pH = 4 (a test for Cl^- ions was negative), aqueous acetic acid (pH 2.3, 300 ml of a 1.0M solution) until the eluate had constant pH = 2.3, and finally with water (550 ml) until neutral. The formate form was prepared in an identical manner by using sodium formate–formic acid washings at the same concentrations. Dowex 50W-X8 (from B.D.H. Chemicals Limited) was prepared in the pyridinium form by washing a column of the acid form with aqueous 2.5M pyridine solution (pH 7.5) until the washings had constant pH and then with water until neutral. Triethylammonium hydrogen carbonate buffer solutions were prepared by bubbling CO_2 through a stirred aqueous solution of 0.4M triethylamine (cooled in an ice-bath) until the pH of the solution fell to 7.5. Other solutions were prepared by diluting the 0.4M solution with water. 1,6-Diaminohexane–carbonate buffer solutions (pH 9.5) were prepared in an identical manner from aqueous 0.1M 1,6-diaminohexane solution. Paper chromatography was carried out on Whatman no. 1 paper. Analytical chromatograms were developed using the ammonium molybdate spray for phosphates[31] and the ninhydrin spray (B.D.H. Chemicals Limited) for amines. Analytical and small-scale preparative h.p.l.c. were carried out using a 50—5 μm Nucleosil column (25 cm × 10 mm i.d.). Reversed phase h.p.l.c. was carried out using a 5 μm Spherisorb ODS column (25 cm × 10 mm i.d.) unless otherwise stated.

3,3:20,20-*Bisethylenedithio*-11α-(3-*carboxypropionyloxy*)-*pregn*-4-*ene* (**2**): (11α-*Hydroxyprogesterone Hemisuccinate* 3,20-*Bisethylenethioacetal*).—Boron trifluoride–diethyl ether (3.1 ml, 0.025 mol) was added to a solutuon of 11α-hydroxyprogesterone hemisuccinate[17] (**1**) (4.15 g, 9.6 mmol) in ethane-1,2-

* Bioluminescence was determined by Dr. J. Hughes, Department of Chemical Pathology, St. Mary's Hospital Medical School, Paddington, London, W2 1PG

dithiol (3.2 ml, 39 mmol) at room temperature and the mixture was set aside for 20 min. The resulting gum was then diluted with dichloromethane (40 ml) and the solution was stirred for 1 h. More dichloromethane (100 ml) was then added and the solution was washed with water (1 × 40 ml and 2 × 50 ml) then with aqueous NaCl (2 × 40 ml of a saturated solution) and dried ($MgSO_4$). Removal of the solvent under reduced pressure gave a white solid (5.76 g). Recrystallization from ethyl acetate–hexane gave 11α-*hydroxyprogesterone hemisuccinate* 3,20-*bisethylenethioacetal* (**2**) (4.59 g, 82%) as needles, m.p. 206—209 °C; $\nu_{max.}$(KBr) 3 660—2 400m,br, 1 720s, 1 705s, 1 640w, 1 225m, and 1 165m cm^{-1}; δ($CDCl_3$) 0.90 (3 H, s, 18-H_3), 1.19 (3 H, s, 19-H_3), 1.83 (3 H, s, 21-H_3), 2.66 (4 H, dd, *J* 4 Hz, δ_A 2.62 and δ_B 2.66, $HO_2CCH_2CH_2CO_2$), 3.00—3.52 (4 H, complex m, SCH_2CH_2S), 5.23 (1 H, td, *J* 10 and 5 Hz, 11β-H), and 5.36 (1 H, s, 4-H) (Found: C, 59.5; H, 7.3; S, 21.8. $C_{29}H_{42}O_4S_4$ requires C, 59.75; H, 7.3; S, 22.0%).

3,3:20,20-*Bisethylenedithio*-11α-[3-(*succinimido-oxycarbonyl*)*propionyloxy*]*pregn*-4-*ene* (**3**).—Triethylamine (0.95 ml, 6.84 mmol) and ethyl chloroformate (0.66 ml, 6.84 mmol) were added successively by syringe *via* a rubber septum to a stirred suspension of 11α-hydroxyprogesterone hemisuccinate 3,20-bisethylene thioacetal (**2**) (1.99 g, 3.42 mmol) in acetonitrile (25 ml) at room temperature under nitrogen. The mixture was stirred for a further 5 min during which all the solids dissolved. More triethylamine (0.95 ml, 6.84 mmol) was then added by syringe *via* the rubber septum, followed by a solution of *N*-hydroxysuccinimide (0.79 g, 6.84 mmol) in dry acetonitrile (35 ml) which was added dropwise over 5 min. The reaction mixture was stirred at room temperature under nitrogen for 30 min and was then evaporated under a stream of nitrogen to a volume of *ca.* 10 ml. The resulting white precipitate was partitioned between ethyl acetate (150 ml) and water (50 ml), the two layers were separated, and the ethyl acetate fraction was washed with more water (5 × 15 ml). The aqueous fractions were combined and extracted with more ethyl acetate (60 ml). The ethyl acetate fractions were combined and the solution was washed with water (15 ml) and dried ($MgSO_4$). Removal of the solvent under reduced pressure gave a white solid (1.99 g), recrystallization of which from ethyl acetate–hexane gave 3,3:20,20-*bisethylenedithio*-11α-[3-(*succinimido-oxycarbonyl*)*propionyloxy*]*pregn*-4-*ene* (**3**) (1.46 g, 63%), as needles, m.p. 209.5—212 °C; $\nu_{max.}$(KBr) 1 810m, 1 780m, 1 735s, 1 640w, 1 210s, 1 100s, 1 085m, and 1 075m cm^{-1}; δ($CDCl_3$) 0.88 (3 H, s, 18-H_3), 1.09 (3 H, s, 19-H_3), 1.82 (3 H, s, 21-H_3), 2.52—3.02 (4 H, complex m, $O_2CCH_2CH_2CO_2$), 2.84 (4 H, s, *succinimido*), 3.08—3.52 (4 H, complex m, SCH_2CH_2S) 5.23 (1 H, td, *J* 10 and 5 Hz, 11β-H), and 5.55 (1 H, s, 4-H) (Found: C, 58.0; H, 6.4; N, 2.0; S, 18.5. $C_{33}H_{45}NO_6S_4$ requires C, 58.3; H, 6.7; N, 2.1; S, 18.9%). The residue of the mother liquors from recrystallization (0.69 g) was purified by h.p.l.c., with a mobile phase of ethyl acetate–hexane (2:3), to give two components. The less polar component was 3,3:20,20-*bisethylenedithio*-11α-[3-(*ethoxycarbonyl*)*propionyloxy*]*pregn*-4-*ene* (**18**) (0.33 g, 16%) needles from ethyl acetate–hexane, m.p. 150.5—152 °C; $\nu_{max.}$(KBr) 1 725s, 1 640w, 1 215m, and 1 165s cm^{-1}; δ($CDCl_3$) 0.89 (3 H, s, 18-H_3), 1.18 (3 H, s, 19-H_3), 1.26 (3 H, t, *J* 7 Hz, OCH_2CH_3), 1.83 (3 H, s, 21-H_3), 2.60 (4 H, s, $O_2CCH_2CH_2CO_2$), 3.00—3.56 (4 H, complex m, SCH_2CH_2S), 4.14 (2 H, q, *J* 7 Hz, OCH_2CH_3), 5.20 (1 H, td, *J* 10 and 5 Hz, 11β-H), and 5.54 (1 H, s, 4-H) (Found: C, 61.8; H, 8.0; S, 19.4. $C_{31}H_{46}O_4S_4$ requires C, 61.9; H, 7.6; S, 20.0%). The more polar component was the *N*-succinimido ester (**3**) (61.0 mg, 3%).

3,3:20,20-*Bisethylenedithio*-11α-(4-*hydroxybutyryloxy*)*pregn*-4-*ene* (**4**).—Sodium borohydride (0.304 g, 8 mmol) was added to a solution of the *N*-succinimido ester (**3**) (1.538 g, 2 mole) in tetrahydrofuran (25 ml) and the mixture was stirred at room temperature under nitrogen. After 18 h the mixture was cooled in ice and excess sodium borohydride was destroyed by the dropwise addition of ice–water (30 ml). The aqueous solution was stirred vigorously, diluted with more water (15 ml), and was extracted with ethyl acetate (1 × 50 ml and 2 × 30 ml). The ethyl acetate fractions were combined and the solution was washed successively with water (15 ml), aqueous $NaHCO_3$ (2 × 10 ml of a saturated solution), water (2 × 15 ml), and saturated brine (20 ml), and dried ($MgSO_4$). Removal of the solvent under reduced pressure gave a white solid (1.112 g) which was purified by h.p.l.c. with a mobile phase of acetone–hexane (1:3) to give two components. The major component was 3,3:20,20-*bisethylenedithio*-11α-(4-*hydroxybutyryloxy*)-*pregn*-4-*ene* (**4**) (0.808 g, 71%) needles from ethyl acetate–hexane, m.p. 196—198.5 °C; $\nu_{max.}$(KBr) 3 700—3 100s,br, 1 720s, 1 640w, 1 175m, and 1 060m cm^{-1}; δ($CDCl_3$) 0.88 (3 H, s, 18-H_3), 1.08 (1 H, s, 19-H_3), 1.82 (3 H, s, 21-H_3), 2.36 (2 H, dd, *J* 6 Hz, δ_A 2.28 and δ_B 2.44, $HOCH_2CH_2CH_2CO_2$), 3.04—3.52 (4 H, complex m, SCH_2CH_2S), 3.68 (2 H, t, *J* 6 Hz, $HOCH_2CH_2CH_2CO_2$), 5.20 (1 H, td, *J* 10 and 5 Hz, 11β-H), and 5.54 (1 H, s, 4-H) (Found: C, 60.9; H, 7.9; S, 22.2. $C_{29}H_{44}O_3S_4$ requires C, 61.2; H, 7.8; S, 22.5%). The minor component (30.1 mg, 2.5%) had identical retention times (t.l.c. and h.p.l.c.) and identical i.r. spectrum with the hemisuccinate (**2**).

11α-(4-*Hydroxybutyryloxy*)*pregn*-4-*ene*-3,20-*dione* (**5**).—*N*-Chlorosuccinimide (0.576 g, 4.32 mmol, freshly recrystallized from benzene, m.p. 148.5—149.5 °C) and silver nitrate (0.826 g, 4.86 mmol) were added to a rapidly stirred suspension of 3,3:20,20-bisethylenedithio-11α-(4-hydroxybutyryloxy)pregn-4-ene (**4**) (0.612 g, 1.08 mmol) in acetonitrile (60 ml) and water (15 ml) at room temperature. Within 2 min the solids and supernatant solution became pale yellow. The reaction mixture was stirred for 20 min and was then treated successively at 1 min intervals with saturated aqueous sodium sulphite (3 ml), saturated aqueous sodium carbonate (3 ml), and saturated brine (3 ml) with rapid stirring. Dichloromethane (50 ml) was then added and the mixture was filtered through Celite 535 [7 g, previously washed with diethyl ether (3 × 50 ml) and dried overnight *in vacuo* at 45 °C]. The Celite pad was then washed with more dichloromethane (2 × 50 ml and 2 × 25 ml), the dichloromethane washings were combined with the original filtrate and the solution was washed with water (3 × 30 ml) then with saturated brine (1 × 25 ml and 2 × 15 ml) and dried ($MgSO_4$). Removal of the solvent under reduced pressure gave a pale-yellow gum (0.362 g). The crude product was purified by reversed phase h.p.l.c. with a mobile phase of water–acetonitrile (1:1) to give one major component and two very minor components. The major component was the required 11α-(4-*hydroxybutyryloxy*)*pregn*-4-*ene*-3,20-*dione* (**5**) (0.236 g, 52%) which separated as a white powder from ethyl acetate–hexane, m.p. 158.5—161 °C; $\nu_{max.}$(KBr) 3 700—3 100s,br, 1 725s, 1 700s, 1 660s, 1 615m, 1 250m, 1 170m, and 1 065m cm^{-1}; $\nu_{max.}$($CHCl_3$) 3 700—3 200m, 1 725s, 1 700s, and 1 615 cm^{-1}; δ($CDCl_3$) 0.71 (3 H, s, 18-H_3), 1.23 (3 H, s, 19-H_3), 2.07 (3 H, s, 21-H_3), 2.16—2.64 (3 H, complex m, $HOCH_2CH_2CH_2CO_2$ and 17α-H), 3.66 (2 H, t, *J* 6 Hz, $HOCH_2CH_2CH_2CO_2$), 5.23 (td, *J* 10 and 5 Hz, 11β-H), and 5.72 (1 H, s, 4-H) (Found: C, 72.30; H, 8.6. $C_{25}H_{36}O_5$ requires C, 72.1; H, 8.7; O, 19.2%). The two minor components, both more polar than the 3,20-dione (**5**), accounted for less than 10% by weight of the purified product and were not investigated further.

3-(3,20-*Dioxopregn*-4-*en*-11α-*yloxycarbonyl*)*propyl Dihydrogen Phosphate* (**6**).—A solution of 11α-(4-hydroxybutyryloxy)pregn-4-ene-3,20-dione (**5**) (0.214 g, 0.51 mmol) and triethylamine (110 μl, 0.78 mmol) in tetrahydrofuran (7 ml) was added using a syringe *via* a rubber septum to a stirred solution

of phosphoryl chloride (71 μl, 0.77 mmol) in tetrahydrofuran (3 ml) at room temperature under nitrogen. The mixture was stirred at room temperature under nitrogen for 3 h and was then evaporated to dryness under a stream of nitrogen. The residual gum was taken up in hexane (5 ml) and the solution was evaporated once more under a stream of nitrogen. The residual gum was then dissolved in ethyl acetate (25 ml) and the solution was washed with aqueous $NaHCO_3$ (1.5% w/v solution; 5 ml) and then with water (4 × 5 ml), and dried ($MgSO_4$). Removal of the solvent under reduced pressure gave a brittle, pale-yellow solid (0.174 g, 63.5%). The crude product, m.p. 98—101 °C, was assigned as a mixture of 3-(3,20-dioxopregn-4-en-11α-yloxycarbonyl)propyl phosphorodichloridate (**19**), 3-(3,20-dioxopregn-4-en-11α-yloxycarbonyl)propyl dihydrogen phosphate (**6**) and a trace of unchanged starting material (**5**); $\nu_{max.}$(KBr) 3 700—3 100s, 2 500—2 220w,br, 1 725s, 1 700s, 1 665s,br, 1 615m, 1 245s (P=O), 1 175s (C–O), 1 065—1 000s (P–O–C), and 970s cm^{-1}; δ($CDCl_3$) 0.71 (3 H, s, 18-H_3), 1.26 (3 H, s, 19-H_3), 2.11 (3 H, s, 21-H_3), 2.20—2.70 [3 H, complex m, $(X)_2P(O)OCH_2CH_2CH_2CO_2$ and 17α-H], 3.60 (*ca.* 0.25 H, t, *J* 6 Hz, $HOCH_2CH_2CH_2CO_2$, *ca.* 15% of mixture), 4.07 (2 H, t, *J* 6 Hz, $(X)_2P(O)OCH_2CH_2CH_2CO_2$], 5.26 (1 H, td, *J* 10 and 5 Hz, 11β-H), and 5.78 (1 H, s, 4-H).

The crude product above (0.163 g, 0.31 mmol) was dissolved in tetrahydrofuran (13.5 ml) and water (1.5 ml) and the solution was stirred and heated under reflux for 3 h under nitrogen; it was then allowed to cool under nitrogen. Removal of the solvent under reduced pressure gave a brittle, pale-yellow solid (0.161 g), which was purified by reversed phase h.p.l.c. with a mobile phase of aqueous 0.01M NaCl–acetonitrile (1:1). Two components were isolated; a major fraction containing organic phosphate (0.138 g, which included *ca.* 35—40 mg of NaCl) and a minor component (0.055 g, which included *ca.* 30—35 mg of NaCl) and which did not contain organic phosphate. The residue from the major fraction above (0.138 g) was dissolved in water (2.5 ml) and ethanol (0.5 ml) and the solution was passed through a column of Dowex 50W-X8, H^+ form, (12 cm × 1.45 cm i.d., 20 ml volume). The column was run with water–ethanol (9:1) as the mobile phase until organic phosphate was no longer detected. Fractions containing organic phosphate were combined and freeze-dried to give 3-(3,20-*dioxopregn*-4-*en*-11α-*yloxycarbonyl*)*propyl dihydrogen phosphate* (**6**) as a glass (0.088 g) which separated from ethyl acetate–hexane as a white powder (0.068 g, 27%), m.p. 112—116 °C; $\nu_{max.}$(KBr) 3 700—3 100s, 1 725s, 1 700s, 1 665s,br, 1 615m, 1 245s (P=O), 1 175s (C–O), 1 065—1 000s (P–O–C), and 970m cm^{-1}; δ($CDCl_3$) 0.71 (3 H, s, 18-H_3), 1.26 (3 H, s, 19-H_3), 2.11 (3 H, s, 21-H_3), 2.20—2.70 [3 H, complex m, $(HO)_2P(O)OCH_2CH_2CH_2CO_2$ and 17α-H], 4.08 [2 H, t, *J* 6 Hz, $(HO)_2P(O)OCH_2CH_2CH_2CO_2$], 5.26 (1 H, td, *J* 10 and 6 Hz, 11β-H), and 5.7 (1 H, s, 4-H). This product was subsequently shown to be homogeneous by reversed phase h.p.l.c. using a μ-Bondapak C-18 column (30 cm × 3.9 mm i.d.) with a mobile phase of aqueous 0.1M NaH_2PO_4–methanol (2:3) and aqueous 0.1M NaH_2PO_4–methanol (1:1). The residue from the minor fraction (0.055 g) was partitioned between ethyl acetate (10 ml) and water (5 ml). The ethyl acetate solution was dried ($MgSO_4$) and the solvent was removed under reduced pressure to give a white solid (21.4 mg) which was assigned as a mixture of starting material (**5**) and 11α-(4-chlorobutyryloxy)pregn-4-ene-3,20-dione (**20**) on the basis of t.l.c. and n.m.r. evidence; δ($CDCl_3$) 0.72 (3 H, s, 18-H_3) and 0.75 (3 H, s, 18-H_3), 1.27 (3 H, s, 19-H_3) and 1.32 (3 H, s, 19-H_3), 2.10 (3 H, s, 21-H_3) and 2.12 (3 H, s, 21-H_3), 2.20—2.82 (2 H, complex m, $XCH_2CH_2CH_2CO_2$, 17α-H and ring CH_2s), 3.60 (2 H, t, *J* 6 Hz, $XCH_2CH_2CH_2CO_2$), 5.22 (1 H, m, 11β-H), and 5.73 (1 H, s, 4-H).

3-(3,20-*Dioxopregn*-4-*en*-11α-*yloxycarbonyl*)*propyl Hydrogen Pyridinium Phosphate* (**7a**).—A solution of the dihydrogen phosphate (**6**) (30.1 mg, 0.06 mmol) in water (4 ml) and ethanol (1 ml) was applied to a column of Dowex 50W-X8 prepared in the pyridinium form[27] (5 cm × 1 cm i.d., 4 ml volume). The column was eluted with water–ethanol (9:1) as the mobile phase until organic phosphate was no longer detected[31] in the eluate (15 ml). The fractions containing the organic phosphates were combined and freeze-dried to give the pyridinium salt (**7a**) as a glass (34.9 mg).

Reaction of Disodium Adenosine-5′-triphosphate with Diphenyl Chlorophosphate.—A solution of disodium adenosine-5′-triphosphate (**8**) (55.1 mg, 0.1 mmol) in water (2 ml) was applied to a column of Dowex 50W-X8 prepared in the pyridinium form[27] (5 cm × 1 cm i.d., 4 ml volume). The column was eluted with water until organic phosphate was no longer detected[31] in the eluate (10 ml). The fractions containing the adenosine-5′-triphosphate pyridinium salt were combined, diluted to 12 ml with water, and tributylamine (26.5 μl, 0.11 mmol) was added. The mixture was stirred at room temperature for 15 min and then the solvent was removed under reduced pressure at room temperature. The residue was then dried by repeated addition and evaporation, under reduced pressure at room temperature, of dry pyridine (3 × 10 ml) and then dry dimethylformamide (4 × 10 ml). The anhydrous tributylammonium salt of adenosine-5′-triphosphate was then dissolved in dry dimethylformamide (2 ml). Diphenylchlorophosphate (0.1 ml, 0.5 mmol) and tributylamine (26.5 μl, 0.11 mmol) were added and the reaction mixture was stirred at room temperature under nitrogen for 20 h. The solvent and excess of diphenylchlorophosphate were removed under reduced pressure and the residual gum was stirred with dry diethyl ether (10 ml) at room temperature for 5 min and then kept at 4 °C for 1 h. The supernatant solution was then decanted and the residue was washed with more dry diethyl ether (4 × 5 ml). Final traces of diethyl ether were removed under reduced pressure to give the activated derivative (**9**) of adenosine-5′-triphosphate (63.2 mg) as its tributylammonium salt, a white crystalline solid; $\nu_{max.}$(KBr) 3 700—2 500s,br, 2 975m, 2 904m, 2 880w, 1 695s, 1 660—1 620m, 1 470m, 1 420m, 1 260s,br, 1 100—1 070m,br, 1 060—1 000m,br, and 935—905m,br cm^{-1}; δ[$(CD_3)_2SO$] 0.88 (3 H, t, *J* 6 Hz, $HN^+CH_2CH_2CH_2CH_3$), 1.02—1.80 (4 H, m, complex m, $HN^+CH_2CH_2CH_2CH_3$), 2.94 (2 H, m, $HN^+CH_2CH_2CH_2CH_3$), 3.72—4.36 (4 H, complex, 5′-H_2, 3′-H, 2′-H), 4.50 (1 H, m, 4′-H), 5.94 (1 H, d, *J* 6 Hz, 1′-H), 6.84—7.24 (10 H, complex m, C_6H_5O), 7.92 (1 H, s, N=CHN), and 8.60 (1 H, s, =NCH=N).

Ammonium Trihydrogen P^1-[3-(3,20-*Dioxopregn*-4-*en*-11α-*yloxycarbonyl*)*propyl*]-P^4-*adenosine*-5′-*tetraphosphate* (**10**).—Tributylamine (7.5 μl, 0.031 mmol) was added to a solution of 3-(3,20-dioxopregn-4-en-11α-yloxycarbonyl)propyl hydrogen pyridinium phosphate (**7a**) (15.7 mg, 0.027 mmol) in water (0.5 ml). The mixture was stirred at room temperature for 10 min and then the solvent was removed under reduced pressure at room temperature. The residue was then dried by repeated addition and evaporation, under reduced pressure at room temperature, of dry dimethylformamide (4 × 10 ml). The anhydrous 3-(3,20-dioxopregn-4-en-11α-yloxycarbonyl)propyl hydrogen tributylammonium phosphate (**7b**) was then dissolved in dry dimethylformamide (0.6 ml) and the solution was stirred at room temperature under nitrogen. A solution of the activated derivative (**9**) of adenosine-5′-triphosphate (29.5 mg, 0.04 mmol) in dry dimethylformamide (2.35 ml) was then added and the mixture was stirred at room temperature under nitrogen for 10 days. A fine white suspension was obtained. Water (1 ml) was added to dissolve the solids and the solvent was then removed under reduced pressure at room temperature

to give a white solid (48.4 mg). The crude product was dissolved in water (1 ml) and ethanol (0.5 ml) and the solution was applied to a column of diethylaminoethyl cellulose DE-52 (12.5 cm × 1 cm i.d., 12 ml volume) prepared in the HCO_3^- form. The column was eluted with water–ethanol (1:1) (9 ml, fractions 1—3) followed by water (24 ml, 2 column volumes, fractions 4—15) and then with a gradient of aqueous 0.1M NH_4HCO_3 to 0.5M NH_4HCO_3. Fractions (2 ml) were collected according to the following scheme; 0.1M NH_4HCO_3 fractions 16—28, 0.2M NH_4HCO_3 fractions 29—38, 0.3M NH_4HCO_3 fractions 39—55, 0.4M NH_4HCO_3 fractions 56—70 and 0.5M NH_4HCO_3 fractions 71—75. The fractions were examined by paper chromatography with isobutyric acid–conc. aq. NH_3–water (66:1:33, v/v) as eluant. Fractions which showed single spots with identical R_F values were combined and the solvent was removed under reduced pressure at room temperature. The NH_4HCO_3 buffer was removed by repeated addition and evaporation of water under reduced pressure at room temperature to constant weight. Fractions 35—36 contained inorganic phosphate. Fractions 39—40 contained unchanged steroid phosphate (**6**), as its ammonium salt (3.2 mg). Fractions 41—43 contained the required conjugate (**10**) (4.2 mg), which was filtered and freeze-dried to give a glass (3.8 mg, 14%); δ(D_2O) 0.53 (3 H, s, 18-H_3), 1.11 (3 H, s, 19-H_3), 2.02 (3 H, s, 21-H_3), 2.12—2.72 (2 H, complex m, P(O)OCH_2CH_2C$H_2$$CO_2$, 17α-H, and ring CH_2s), 3.74 (2 H, t, *J* 6 Hz, P(O)OCH_2CH_2CH_2-CO_2), 3.92 (2 H, dd, *J* 6 Hz and *J* 3 Hz, 5′-H_2), 4.10—4.40 (2 H, complex m, 3′- and 2′-H), *ca.* 4.50 (1 H, shoulder on HOD peak, 4′-H), 4.92—5.28 (1 H, m, 11β-H), 5.64 (1 H, s, 4-H), 5.95 (1 H, d, *J* 6 Hz, 1′-H), 8.06 (2 H, s, A8-H, N=CHN), and 8.32 (2 H, s, A2-H, =NCH=N–) (Found: C, 40.8; H, 5.4; N, 8.5; P, 12.5. $C_{35}H_{50}N_5P_4O_{20}^-NH_4^+ \cdot H_2O$ requires C, 41.2; H, 5.5; N, 8.2; P, 12.1%). Fractions 45 and 46, glass (1.6 mg), fractions 49—53, hygroscopic glass (6.0 mg), fractions 55—61, glass (6.9 mg), and fractions 63—65, glass, hygroscopic (2.5 mg) were all shown by n.m.r. (D_2O) to be non-steroidal. These compounds were assigned as adenosine-5′-mono-, di-, or tri-phosphates but were not investigated further.

11α-[3-(*Succinimido-oxycarbonyl*)*pregn*-4-*ene*-3,20-*dione* (**11**).—Triethylamine (1.3 ml, 9.3 mmol) and ethyl chloroformate (0.9 ml, 9.3 mmol) were added successively by syringe *via* a rubber septum to a stirred suspension of 11α-hydroxyprogesterone hemisuccinate (**1**) (2.0 g, 4.65 mmol) in acetonitrile (20 ml) at room temperature under nitrogen. The mixture was stirred for a further 5 min during which all of the solids dissolved. More triethylamine (1.3 ml, 9.3 mmol) was then added by syringe *via* a rubber septum, followed by a solution of *N*-hydroxysuccinimide (1.07 g, 9.3 mmol) in acetonitrile (30 ml) dropwise over a period of 6 min. The reaction mixture was stirred at room temperature under nitrogen for 1 h and was then evaporated under a stream of nitrogen to a volume of *ca.* 10 ml. The resulting white precipitate was partitioned between ethyl acetate (75 ml) and water (30 ml). The ethyl acetate layer was washed with water (1 × 15 ml and 2 × 10 ml). The ethyl acetate layer was washed with water (1 × 15 ml and 2 × 10 ml) and dried ($MgSO_4$). Removal of the solvent under reduced pressure gave a yellow gum, which partially solidified (2.44 g). The crude product was purified by h.p.l.c. using a 'PrepPAK' 500 silica column with a mobile phase of ethyl acetate–hexane (3:1) to give two components. The major product was 11α-[3-(succinimido-oxycarbonyl)propionyloxy]pregn-4-ene-3,20-dione (**11**) (1.42 g, 60%) as needles from ethyl acetate–hexane, m.p. 191—194 °C (lit.,[32] 195—197 °C); $\nu_{max.}$(KBr) 1 810m, 1 785, 1 735s, 1 700s, 1 670s, 1 615w, 1 265m, 1 200s, 1 090m, and 1 075m cm^{-1}; δ($CDCl_3$) 0.73 (3 H, s, 18-H_3), 1.28 (3 H, s, 19-H_3), 2.11 (3 H, s, 21-H_3), 2.50—3.08 (4 H, complex m, $O_2CCH_2CH_2CO_2$), 2.83 (4 H, s, succinimido-H), 5.30 (1 H, td, *J* 10 and 5 Hz, 11β-H), and 5.74 (1 H, s, 4-H) (Found: C, 66.2; H, 7.3; N, 2.6. Calc. for $C_{29}H_{37}NO_8$: C, 66.0; H, 7.1; N, 2.65%). The minor product which was less polar than the *N*-succinimido ester (**11**) was isolated as a gum (0.32 g) which, with time, slowly formed needles. Analytical h.p.l.c. of this material using a 10 μm Partisil column (25 cm × 3.9 mm i.d.) with a mobile phase of ethyl acetate–hexane (3:2) showed two components in a ratio of 7:3 which have not been investigated further.

8-(6-*Aminohexyl*)*aminoadenosine*-5′-*triphosphate* (**12**).—This was prepared following the procedure of Trayer *et al.*[27] as a white powder; $\nu_{max.}$(KBr) 3 700—2 300s,br, 2 940m, 1 680s, 1 650s, 1 615s, 1 515w, 1 255—1 245s, 1 135s, 1 085s, 915s, and 810m cm^{-1}; δ(D_2O) 1.04—1.76 [8 H, complex m, $HNCH_2$-$(CH_2)_4CH_2NH_2$], 2.86 (2 H, t, *J* 7 Hz, CH_2NH_2), 3.32 (2 H, t, *J* 7 Hz, $NHCH_2$), 4.19 (2 H, m, 5′-H_2), 4.28—*ca.* 4.80 (3 H, m, 2′-, 3′-, and 4′-H partially hidden by the HOD peak), 5.86 (1 H, d, *J* 7.5 Hz, 1′-H), and 8.0 (2 H, br s, A2-H, =NCH=N); $\lambda_{max.}$(H_2O) 276 nm (ε 14 300); $\lambda_{min.}$ 235 nm.

Analytical h.p.l.c. using a Nucleosil 5 SB anion exchange column (25 cm × 3.9 mm i.d.) with a mobile phase of aqueous 0.6M KH_2PO_4 showed the product to be >92% pure. The impurity which eluted before the major peak might be 8-(6-aminohexyl)aminodenosine-5′-diphosphate.

8-(6-*Aminohexyl*)*amino*-β-*nicotinamide Adenine Dinucleotide* (**13**).—This was prepared by a method based upon the procedure of Lee *et al.*,[28] as a very pale yellow powder; $\nu_{max.}$ 3 700—2 400s,br, 2 940m, 1 685m, 1 645—1 610s, 1 590m, 1 470w, 1 340w, 1 240s, 1 120s, 1 080s, 940s, and 825—805m cm^{-1}; δ(D_2O) 1.02—1.80 [8 H, complex m, $HNCH_2(CH_2)_4$-CH_2NH_2], 2.82 (2 H, t, *J* 7 Hz, CH_2NH_2), 3.25 (t, *J* 7 Hz, $NHCH_2$), 3.80—*ca.* 4.60 (5 H, complex m, 5′-H_2, 3′-, 2′-, and 4′-H partially hidden by the HOD peak), 5.73 (2 H, d, *J* 7.5 Hz, 1′-H, NC*H*), 5.93 (2 H, d, *J* 4 Hz, 1′-H, $=N^+CH$), 7.80 (1 H, s, A2-H), 8.04 (1 H, t, *J* 7 Hz, N5-H), 8.66 (1 H, d, *J* 7 Hz, N4-H), 9.01 (1 H, d, *J* 7 Hz, N6-H), and 9.17 (1 H, s, N2-H); $\lambda_{max.}$(H_2O) 273 nm (ε 18 800); $\lambda_{min.}$ 238; *m/z* 779 ([*M* + H]$^+$, 9%), 778 (M^+, 19%), 656 (3), 542 (5), 279 (6), 278 (3), 263 (6), 251 (14), 250 (100), 237 (11), 221 (20), and 97 (54).

The preparative sequence for this nucleotide is described below.

8-*Bromo*-β-*nicotinamide Adenine Dinucleotide* (**21**).—Bromine (0.69 ml, 13.5 mmol) was added using a syringe *via* a rubber septum in 4 aliquots of 172.5 μl at intervals of 30 min to a stirred solution of β-nicotinamide adenine dinucleotide (0.699 g, 1.0 mmol) in 1.0M aqueous sodium acetate–acetic acid buffer (pH 4.0; 20 mol) at room temperature under nitrogen in the dark. The reaction mixture was stirred for 20 h under these conditions and was then diluted with water (100 ml). The aqueous solution was cooled in an ice-bath, stirred thoroughly, and made alkaline by addition of aqueous NaOH to pH 8 (1.0M solution; 3 ml). The solvent was removed under reduced pressure at room temperature to leave a yellow gum (4.42 g). The crude product was dissolved in water (10 ml) and the solution was applied to a column of Dowex 1-X8, 100—200 mesh (64 cm × 2.0 cm i.d., 200 ml volume) prepared in the AcO^- form. The column was eluted with water (300 ml, 1.5 column volumes, fractions 1—15) and then with a gradient of aqueous 0.1M AcOH to 0.7M AcOH. Fractions were collected according to the following scheme; 0.1M AcOH fractions 16—25, 0.2M AcOH fractions 36—30, 0.3M AcOH fractions 31—35, 0.4M AcOH fractions 36—40, 0.5M AcOH fractions 41—45, 0.6M AcOH fractions 46—50, and 0.7M AcOH fractions 51—70. The product was detected by u.v. absorbance at 254 nm, and by the

ammonium molybdate spray,[31] in fractions 50—66. These fractions were combined and the solution was freeze-dried to give 8-bromonicotinamide adenine dinucleotide (0.212 g, 28.5%) as a white power; δ(D_2O) 4.00—4.72 (5 H, complex 5′-H_2, 3′-, 2′-, and 4′-H, partially hidden by the HOD peak), 6.01 (2 H, d, *J* 6 Hz, 1′-H, NCH), 6.07 (2 H, d, *J* 3 Hz, 1′-H, $=^+$NCH), 8.18 (1 H, t, *J* 7 Hz, N5-H), 8.28 (1 H, s, A2-H), 8.82 (1 H, d, *J* 7 Hz, N4-H), 9.16 (1 H, d, *J* 7 Hz, N6-H), and 9.31 (1 H, s, N2-H); $\lambda_{max.}$ 264 nm (ε 16 180); $\lambda_{min.}$ 229 nm; *m/z* 744 ([*M* + H]$^+$, 18%), 743 (M^+, 9%), 506 (10), 329 (100), and 328 (53).

This reaction was repeated on the same scale and the products from the two reactions were combined.

8-(6-*Aminohexyl*)*amino*-β-*nicotinamide Adenine Dinucleotide* (**13**).—8-Bromo-β-nicotinamide adenine dinucleotide (0.37 g, 0.5 mmol) was dissolved in aqueous 0.1M 1,6-diaminohexane carbonate buffer solution (pH 9.5, 25 ml). Ethanol (5.9 ml, 0.1 mol) was then added using a syringe *via* a rubber septum and a stream of nitrogen was passed slowly through the stirred solution at 25 °C. A solution of yeast alcohol dehydrogenase (125 μl of a solution containing 5.17 mg of solid ≡ 4.81 mg of protein in 0.4 ml water, 451 units) was then added using a syringe *via* a rubber septum. The mixture was stirred at 35 °C and the nitrogen outlet was passed into a stirred solution of semicarbazide hydrochloride (0.112 g, 1 mmol) in 0.1M potassium pyrophosphate buffer (20 ml). The reaction was followed by removing aliquots of the reaction mixture (25 μl), diluting each to 0.5 ml with water, and measuring the increase in absorbance at 340 nm. The reaction was complete after 10 min; $\lambda_{max.}$ 265 (ε 14 260) and 340 nm (ε 5 550). The solvent was removed under reduced pressure at room temperature and the residue was dissolved in dry dimethyl sulphoxide (25 ml). 1,6-Diaminohexane (1.74 g, 15 mmol) was added and the mixture was stirred and heated under nitrogen at 60 °C (oil-bath). The reaction was followed by allowing the reaction mixture to cool under nitrogen, removing aliquots (20 μl), diluting each to 0.5 ml with water, and measuring the u.v. spectrum, until there was no further shift in the absorbance maximum from 264 to 277 nm. The reaction was complete after 6 h. The solvent was then removed under reduced pressure at room temperature. The residue was dissolved in water (10 ml) and applied to a column of diethylaminoethyl cellulose DE-52 (32 cm × 2.0 cm i.d. 100 ml volume) prepared in the HCO_3^- form. The column was eluted with water (100 ml, 1 column volume, fractions 1—10) and then with a gradient from aqueous 0.1M NH_4HCO_3 to 1.0M NH_4HCO_3 solution which was increased in 0.1M steps at intervals of 1 column volume. The fractions collected were examined by u.v. and with the ammonium molybdate spray. The fractions with u.v. absorption at 254 and 350 nm were combined and the solvent was removed under reduced pressure at room temperature. The NH_4HCO_3 buffer was removed by repeated addition and evaporation of water under reduced pressure at room temperature to constant weight. The residue was a glass [73.9 mg, 19% based upon 8-(6-aminohexyl)amino-β-NADH].

This material [46.6 mg, 0.06 mmol, based upon 8-(6-aminohexyl)amino-β-NADH] was dissolved in aqueous 0.1M ammonium hydrogen carbonate buffer solution (pH 7.5; 20 ml) at room temperature. Acetaldehyde (1.0M solution in water; 12 ml, 12 mmol) and a solution of yeast alcohol dehydrogenase (15 μl of a solution containing 5.17 mg of solid ≡ 4.81 mg of protein in 0.5 ml H_2O, 54 units) were then added successively using a syringe *via* a rubber septum and the mixture was stirred at 25 °C. The reaction was followed by removing aliquots of the reaction (200 μl), diluting each with water to 0.5 ml, and measuring the decrease in absorbance at 340 nm. After 1 h, the solvent and excess of acetaldehyde were removed under reduced pressure at room temperature. The NH_4HCO_3 buffer was removed by repeated addition and evaporation of water under reduced pressure at room temperature to give a pale yellow solid (48.3 mg). The residue was then chromatographed using a column of diethylaminoethyl cellulose DE-52 (32 cm × 2.0 cm i.d. 100 ml volume) prepared in the HCO_3^- form following the procedure described above. The fractions collected were examined by paper chromatography using isobutyric acid–conc. aq. NH_3–water (66:1:33, by volume) as eluant. One major fraction was detected by its u.v. absorption at 254 nm and with the ammonium molybdate spray; it gave a very weak positive response to the ninhydrin spray. These fractions were combined and worked-up as previously described to give 8-(6-aminohexyl)amino-β-nicotinamide adenine dinucleotide (**13**) (32.7 mg) as a pale yellow solid.

N^6-(6-*Aminohexyl*)*carbamoylmethyl*-β-*nicotinamide Adenine Dinucleotide* (**14**).—This was purchased from Sigma Chemical Company Limited, Poole, Dorset and was used without further purification; $\nu_{max.}$ 3 700—2 000s,br, 2 960w, 2 940w, 1 690m, 1 650m,sh, 1 640m,sh, 1 620s, 1 585w, 1 475w, 1 405w, 1 335w, 1 240s, 1 115s, 1 075s, 945m, and 825—795m,br cm^{-1}; δ(D_2O) 0.72—1.56 [8 H, complex m, $HNCH_2(CH_2)_4CH_2NH_2$], 2.75 (2 H, t, *J* 8 Hz, CH_2NH_2), 3.04 (2 H, t, *J* 7 Hz, $NHCH_2$), 4.09 [2 H, s, $HNCH_2C(O)NH$], 4.10—4.90 (5 H, complex m, 5′-H_2, 3′-, 2′-, and 4′-H partially hidden by the HOD peak), 5.91 (2 H, d, *J* 7 Hz, 1′-H, NC*H*), 5.97 (2 H, d, *J* 4 Hz, 1′-H, $=^+$NCH), 8.08 (2 H, s, A2-H and t, *J* 7 Hz, N5-H), 8.30 (1 H, s, A8-H), 8.72 (1 H, d, *J* 7 Hz, N4-H), 9.05 (1 H, d, *J* 7 Hz, N6-H), and 9.21 (1 H, s, N2-H); $\lambda_{max.}$(H_2O) 263.5 nm (ε 22 000); $\lambda_{min.}$ 229 nm; *m/z* 821 ([*M* + H]$^+$, 4.5%), 820 (M^+, 10%), 698 (1), 584 (4), 292 (10), 223 (44), 222 (7), 221 (15), 176 (6), 148 (10), 115 (100), and 97 (24).

General Procedure for the Preparation of the Conjugates (**15**)—(**17**) *of* 11α-*Hydroxyprogesterone with ATP and* β-*NAD.*—*Reaction of* 11α-*hydroxy*-3-(*succinimido-oxycarbonyl*)*propionyl-oxy*]*pregn*-4-*ene*-3,20-*dione* (**11**) *with* 8-(6-*aminohexyl*)-*aminoadenosine*-5′-*triphosphate* (**12**), 8-(6-*aminohexyl*)*amino*-β-*nicotinamide adenine dinucleotide* (**13**), *and* N^6-[(6-*aminohexyl*)-*carbamoylmethyl*]-β-*nicotinamide adenine dinucleotide* (**14**).—The nucleotide (0.01 mmol) was dried by repeated evaporation of its solution in dry dimethyl sulphoxide (5 × 1 ml) under reduced pressure at room temperature until the residue had constant weight. The residue was then dissolved in dry dimethyl sulphoxide (1.5 ml) and the solution was stirred at room temperature under nitrogen. Triethylamine (5.5 μl, 0.04 mmol, and then a solution of the *N*-succinimido ester (**11**) (7.9 mg, 0.015 mmol) in dry dimethyl sulphoxide (0.5 ml) were added using syringes *via* a rubber septum. The reaction mixture was then stirred at room temperature under nitrogen in the dark for 6 days. The solvent was removed under reduced pressure at room temperature to leave a residual gum which was taken up in water (15 ml). The aqueous solution was extracted with dichloromethane (1 × 5 ml and 3 × 1 ml) and the extracts were combined ($MgSO_4$), and evaporated under reduced pressure to give a colourless glass (0.5—0.7 mg) which was shown by t.l.c. to be a mixture of 11α-hydroxyprogesterone hemisuccinate (**1**) and the *N*-succinimido ester (**11**). The aqueous solution was examined by paper chromatography using butan-1-ol–acetic acid–water (5:2:3, v/v) as eluant. From all three reactions a similar pattern was observed; excess of steroid [as 11α-hydroxyprogesterone hemisuccinate (**1**)]; R_F > 0.9, *N*-hydroxy-succinimide R_F 0.65, and the product conjugates, (**15**), R_F 0.47; (**16**), R_F 0.41; and (**17**), R_F 0.46. None of the starting nucleotides were detected in the product mixtures. The aqueous solutions were freeze-dried to give a colourless or pale yellow glass (16.1—17.1 mg). The crude products were purified by preparative paper chromatography with a freshly prepared mixture of butan-1-ol–

acetic acid–water (5:2:3, v/v) as the eluant. The samples were dissolved in water (500 μl) and were loaded on to 20 cm × 20 cm squares of chromatography paper as spots (10 spots per sheet 1.5 cm apart, loading 80 μg per spot). The solvent rise was 15 cm. The conjugates were washed from the paper with water and the solutions were freeze-dried. To characterize the products, each conjugate was dissolved in water–ethanol (1:9) (1—1.5 ml) and the solutions were added to columns of Dowex 50W-X8 (5 cm × 1 cm i.d., 5 ml volume), prepared in the lithium form for the ATP conjugate (**15**) and in the H^+ form for the conjugates (**16**) and (**17**) with NAD. The eluate was monitored by paper chromatography using the ammonium molybdate spray.[31] Fractions were combined as appropriate, and the solutions were freeze-dried to constant weight but the conjugates were not made anhydrous. Extinction coefficients were calculated on the basis of the formula weight for the hydrated conjugates.

Ammonium 8-(10-(3,20-*dioxopregn*-4-*en*-11α-*yloxycarbonyl*-8-*oxo*)-7-*azadecyl*]*aminoadenosine triphosphate* (**15**), a colourless glass (6.3 mg, 58%); $\lambda_{max.}(H_2O)$ 252.5 (ε 18 970) and 277.5 nm (18 000); $\lambda_{min.}$ 234 nm; δ[$(CD_3)_2SO$] 0.69 (3 H, s, 18-H_3), 1.21 (3 H, s, 19-H_3), 2.10 (3 H, s, 21-H_3), 2.10—2.85 [complex m, $HNCOCH_2CH_2CO_2$, 17α-H and ring CH_2s, partly hidden by the $(CD_3)_2SOD_5$ peak], 2.99 (2 H, t, *J* 7 Hz, CH_2NH), 3.25 (2 H, t, *J* 7 Hz, $NHCH_2$), 3.7—4.8 (5 H, complex m, 5′-H_2, 3′-, 2′-, and 4′-H), 5.20 (1 H, m, 11β-H), 5.71 (2 H, s, 4-H and d, *J* 7 Hz, 1′-H), and 7.92 (2 H, br s, A2-H, =NCH=N) (Found: C, 45.4; H, 6.0, Li, 0.8; N, 8.8; P, 8.7. $C_{41}H_{61}LiN_7O_{18}P_3$ requires C, 47.4; H, 5.9; Li, 0.7; N, 9.4; P, 8.9%. $C_{41}H_{61}LiN_7O_{18}P_3 \cdot 3H_2O$ requires C, 45.0; H, 6.2; Li, 0.6; N, 9.0; P, 85%).

8-[10-(3,20-*Dioxopregn*-4-*en*-11α-*yloxycarbonyl*)-8-*oxo*-7-*azadecyl*]*aminonicotinamide adenine dinucleotide* (**16**), a white solid (6.2 mg, 48%); $\lambda_{max.}(H_2O)$ 250 (ε 17 400) and 280 nm (15 700); $\lambda_{min.}$ 240 nm; δ[$(CD_3)_2SO$] 0.68 (3 H, s, 18-H_3), 1.22 (3 H, s, 19-H_3), 2.10 (3 H, s, 21-H_3), 2.15—2.8 [complex m, $HNCOCH_2CH_2CO_2$, 17α-H and ring CH_2's, partially hidden by the [$^2H_5$]DMSO peak], 3.01 (2 H, t, *J* 7 Hz, CH_2NH), 3.20 (2 H, t, *J* 7 Hz, $NHCH_2$), 3.7—4.9 (5 H, complex m, 5′-H, 3′-, 2′-, and 4′-H), 5.18 (1 H, m, 11β-H), 5.70 (3 H, s, 4-H, and d, *J* 7 Hz, 1′-H, NCH), 5.88 (2 H, m, 1′-H, $=N^+CH$), 7.90—8.20 (m, N5-H), 7.92 (s, A2-H), 8.26 (d, *J* 6 Hz, N4-H), 8.94 (d, *J* 6 Hz, N6-H), and 9.08 (1 H, s, N2-H) (Found: C, 49.1; H, 6.3; N, 10.1; P, 5.1. $C_{52}H_{73}N_9O_{19}P_2$ requires C, 52.5; H, 6.2; N, 10.6; P, 5.2%. $C_{52}H_{73}N_9O_{19}P_2 \cdot 5H_2O$ requires C, 48.8; H, 6.5; N, 9.9; P, 4.8%).

N^6-{10-(3,20-*Dioxopregn*-4-*en*-11α-*yloxycarbonyl*)-8-*oxo*-7-*azadecyl*]*carbamoylmethyl*}*nicotinamide adenine dinucleotide* (**17**), a white solid (9.1 mg, 73%); $\lambda_{max.}(H_2O)$ 251—255 nm (ε 22 560); $\lambda_{min.}$ 229 nm; δ[$(CD_3)_2SO$] 0.67 (3 H, s, 18-H_3), 1.22 (3 H, s, 19-H_3), 2.10 (3 H, s, 21-H_3), 2.1—2.9 (complex m, $HNCOCH_2CH_2CO_2$, 17α-H and ring CH_2's, partly hidden by the [$^2H_5$]DMSO peak), 2.95—3.15 (2 H, m, $NHCH_2$) 3.7—4.9 (7 H, complex m, 5′-H_2, 3′-H, 2′-H, 1′-H, and $HNCH_2CONH$), 5.19 (1 H, m, 11β-H), 5.71 (1 H, s, 4-H), 5.75 (2 H, d, *J* 7 Hz, 1′-H, NCH), 5.90 (m, 1′-H, $=N^+CH$), 7.90—8.20 (3 H, m, N5-H, A2-H, and A8-H), 8.26 (1 H, d, *J* 6 Hz, N4-H), 8.95 (1 H, d, *J* 6 Hz, N6-H), and 9.10 (1 H, s, N2-H) (Found: C, 51.5; H, 6.4; N, 10.1; P, 4.6. $C_{54}H_{75}N_9O_{29}P_2$ requires C, 52.6; H, 6.1; N, 10.2; P, 5.0%. $C_{54}H_{75}N_9O_{20}P_2 \cdot 2H_2O$ requires C, 51.1; H, 6.3; N, 9.9; P, 4.9%).

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