The Synthesis of a Conjugate of Progesterone with Lucifer Yellow VS: a Potential Probe for Fluoroimmunoassay of Steroids

David N. Kirk * and Barry W. Miller
Chemistry Department, Queen Mary College, Mile End Road, London, E1 4NS

The synthesis of a conjugate of 11α-hydroxyprogesterone with the 1,3-dioxobenz[*de*]isoquinoline-5,8-disulphonate Lucifer Yellow VS is reported. Preliminary evaluation of fluorescence indicates that the working limit in immunoassay will be solutions of *ca.* 2 nmolar concentration.

Radioimmunoassay of steroids[1] has long been established as a valuable analytical procedure. It does however suffer from a number of problems associated with the use of radioactive materials. Fluoroimmunoassay[2,3] is a relatively inexpensive technique which avoids the problems and hazard of using radioactive materials and has been the focus of much recent interest.

Criteria can be listed for an ideal fluorescent label. It should have a high quantum yield and a large Stokes shift, preferably greater than 100 nm; it should emit light at a wavelength longer than 550 nm; it should be easy to attach to the steroid and should form a stable conjugate. Historically, most fluorescence immunoassays have involved fluorescein, as for example fluorescein isothiocyanate and the fluorescein amines or rhodamine labels. The major drawback with both of these labels is their small Stokes shift, 26 nm for fluorescein isothiocyanate and 35 nm for rhodamine-B200-isothiocyanate. Fluorescein fluorescence is also pH-dependent.[4,5]

Many new and interesting fluorescent labels have been studied.[6–15] One of these is 'Lucifer Yellow VS' (**1**). The Lucifer dyes[16] 'Lucifer Yellow VS' (**1**) and 'Lucifer Yellow CH' (**2**) are stable, strongly fluorescent 6-amino-1,3-dioxobenz[*de*]isoquinoline-5,8-disulphonates † which differ only in the substituent on the imide nitrogen. Both of these compounds have absorption maxima at 280 and 430 nm and an emission maximum at 540 nm.[13,16] Lucifer Yellow CH (**2**) has been used as a fluorescent label for biological tracing[17,18] and Lucifer Yellow VS was similarly used in the determination of albumin in serum.[13]

In this paper we present the synthesis of a derivative of progesterone labelled with Lucifer Yellow VS, dilithium 6-amino-2-{*m*-[3-(3,20-dioxopregn-4-en-11α-yloxycarbonyl)-propylaminoethylsulphonyl]phenyl}-1,3-dioxobenzo[*de*]isoquinoline-5,8-disulphonate (**8**).

Results and Discussion

The vinyl sulphone group of Lucifer Yellow VS (**1**) readily undergoes conjugate addition with soft nucleophiles such as amino and thiol groups and hence can be easily attached to proteins. A simple approach to labelling progesterone with Lucifer Yellow VS is therefore to attach the dye to the steroid *via* an amino acid-like bridge. We chose to use the steroidal 4-aminobutyrate (**7a**).

11α-Hydroxyprogesterone (**3**) was treated with ethane-1,2-diol and toluene-*p*-sulphonic acid in refluxing toluene to give 3,3:20,20-bisethylenedioxypregn-5-en-11α-ol (**4**). Condensation of the 3,20-bisethylene acetal (**4**) with 4-t-butoxycarbonylaminobutyric acid in the presence of di-isopropylcarbodi-imide and a catalytic amount of 4-dimethylaminopyridine gave 3,3:20,20-bisethylenedioxy-11α-(4-t-butoxycarbonylamino-butyryloxy)pregn-5-ene (**5**) in >70% yield. The use of carbodi-imides to construct ester and amide bonds is well established.[19] Recently reactions under conditions of acidic[20] and basic catalysis[21] have led to improved yields in reactions with dicyclohexylcarbodi-imide (DCC). Replacing DCC with di-isopropylcarbodi-imide has led to a more efficient procedure. The only side product, the urea derivative (**6**), was easily separated from the progesterone derivative (**5**).

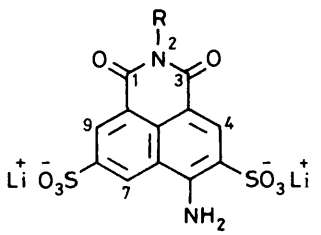
The required amino derivative of progesterone, 11α-(4-amino-butyryloxy)pregn-4-ene-3,20-dione (**7a**) could then be generated by acid hydrolysis of the protected compound (**5**). However, some selectivity in the ease of hydrolysis allowed removal of the acetals while retaining the t-butoxycarbonyl group. The free amine (**7a**) was very unstable and could be stored only in the buffer solution, aqueous 5.0 mM diammonium hydrogen phosphate. A sample retained for t.l.c. in a 1:1 mixture of this buffer and acetonitrile was stable for several weeks when stored in a refrigerator, whereas samples of (**7a**) dissolved in organic solvents quickly became dark brown, t.l.c. of these solutions showing more than one spot.

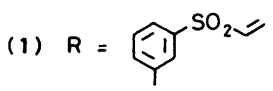
Hydrolysis of the protected compound (**5**) with up to 60 mol equiv. of trifluoroacetic acid and up to 90 mol equiv. of acetic acid in aqueous tetrahydrofuran at room temperature led to cleavage of the ethylene acetal groups only. The resulting (4-t-butoxycarbonylaminobutyryloxy)pregn-4-ene-3,20-dione (**7b**) was isolated quantitatively as a glass. The free amine (**7a**) was generated by acid hydrolysis directly from (**5**), as required, by using a 200-fold molar excess of trifluoroacetic acid in aqueous dioxane. The reaction was followed by t.l.c.: after 20 h at room temperature only a trace of the protected amine (**7b**) remained; this was hydrolysed completely to the free amine (**7a**) by reaction at 60 °C for 1.25 h. T.l.c. of the product on silica, pre-saturated with the mobile phase of 5.0 mM $(NH_4)_2HPO_4$–acetonitrile (1:1), showed a single spot which was strongly ninhydrin-positive.

The freshly isolated free amine (**7a**) was treated immediately with Lucifer Yellow VS (**1**) (1 mol equiv.) in aqueous dioxane in the presence of triethylamine. After 74 h paper chromatography using a mixture of butan-1-ol–acetic acid–water (5:2:3, v/v) as mobile phase showed loss of both reactants and the appearance of a new fluorescent spot. The water-soluble conjugate was isolated as a pale yellow solid. The crude material was washed through a column of Dowex 50W-X8 prepared in the lithium form to allow characterization as the dilithium salt (**8**).

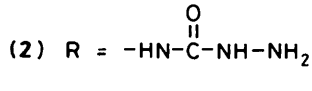
Characterization of this conjugate by ^1H n.m.r. spectroscopy was difficult because of the low solubility and poor spectral resolution in D_2O and $(CD_3)_2SO$, although the spectrum confirmed the presence of both the steroid and the Lucifer Yellow entities. The conjugate is insoluble in organic solvents. The u.v. spectrum, in water, showed absorption assigned to the

† Also called 4-aminonaphthalene-1,8-dicarboximides.

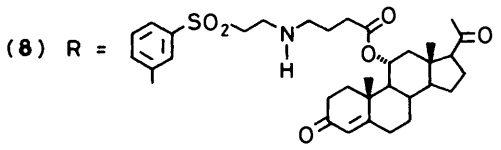




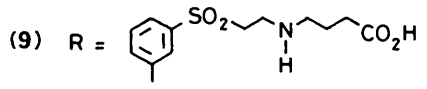
(1) R =



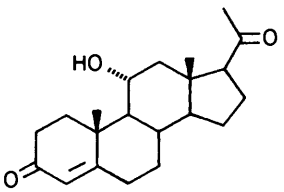
(2) R =



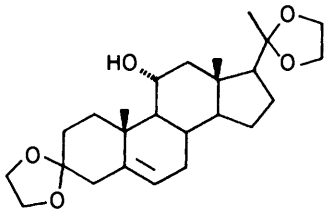
(8) R =



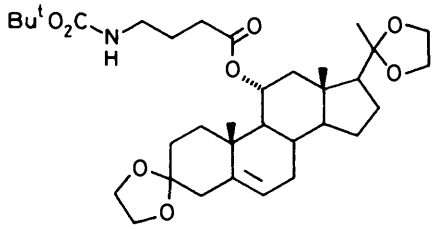
(9) R =



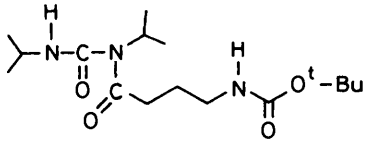
(3)



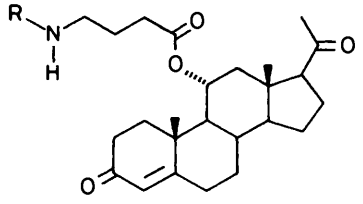
(4)



(5)



(6)



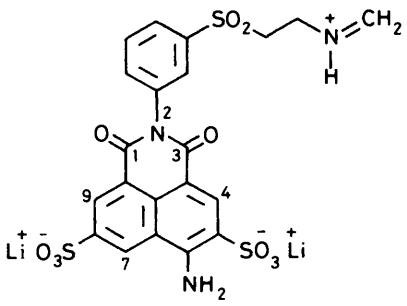
(7) (a) R = H

(b) R = CO_2Bu^t

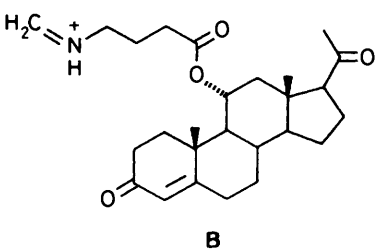
Lucifer Yellow VS chromophore at 432 (ε 10 390), 279.5 (21 550), and 229sh nm (31 420) and the appearance of a new absorption band, an apparent maximum at 257 nm (ε = 19 730) which we assign to the strongly red-shifted steroidal $\pi \rightarrow \pi^*$ transition. In solution in absolute ethanol the extinction coefficients are reduced to between 70% and 80% of their values in water. The absorption at 257 nm undergoes only a small blue shift to 255 nm in ethanol and is much less well resolved within the trough between the Lucifer Yellow maxima at 279 and 227 nm.

The fast atom bombardment (f.a.b.) mass spectrum was recorded for a solution in glycerol. The very low solubility of the conjugate (**8**) in glycerol and thioglycerol caused a high background due to solvent clusters.[22] A weak $[M + H]^+$ peak (m/z 966; 5%) was detected. The characteristic peaks were pairs derived from the Lucifer Yellow fragment, the iminium ion (**A**) (m/z 587 $[\mathbf{A} + Li]^+$ and m/z 581 $[\mathbf{A} + H]^+$) and from the steroidal iminium ion (**B**) (m/z 435 $[B + Li]^+$ and m/z 429 $[B + H]^+$) (Figure). The f.a.b. spectrum of the analogous dilithium 6-amino-2-[*m*-(carboxypropylaminoethylsulphonyl)phenyl]-1,3-dioxobenzo[*de*]isoquinoline-5,8-disulphonate (**9**), prepared from Lucifer Yellow VS (**1**) and 4-aminobutyric acid, also showed peaks at m/z 587 and m/z 581 containing fragment (**A**).

Our initial fluorescence measurements indicate that the conjugate (**8**) can be detected in pure aqueous solution at a



A



B

Figure.

concentration of 2 nM. The working limit in immunoassays would in practice be higher than this value to take account of random scattering and background fluorescence from biological fluids.

Experimental

M.p.s were determined on a Reichert melting point microscope. I.r. spectra were determined for potassium bromide discs unless otherwise stated. N.m.r. spectra were determined at 100 MHz for solutions in deuteriochloroform with tetramethylsilane as internal standard unless otherwise stated. Fast atom bombardment (f.a.b.) mass spectra were recorded on a Kratos MS 902 spectrometer with M-scan source (argon) and MSS data acquisition for solutions in glycerol. All solvents were purified before use.[23] Hexane refers to the light petroleum fraction of boiling range 60—80 °C. Water was distilled from glass and freed from organic contaminants by passage through a 'Norganic' cartridge (Millipore Corporation, Bedford, Massachusetts 017300). Analytical and small scale preparative h.p.l.c. were carried out on a 25 cm × 10 mm i.d. stainless steel column packed with 50-5 μm Nucleosil. Lucifer Yellow VS and 2-(t-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC–ON) were purchased from Aldrich Chemical Company Ltd., Gillingham, Dorset (UK). Ninhydrin spray reagent was purchased from BDH Ltd., Poole, Dorset (UK). Fluorescence spectra were measured using a Perkin-Elmer 3000 spectrophotometer for solutions in water.

Lucifer Yellow VS (Dilithium 6-amino-2-(*m*-vinylsulphonylphenyl)1,3-dioxobenz[*de*]isoquinoline-5,8-disulphonate) (**1**), δ(D_2O) 6.16 (dd, *J* 10 and 1 Hz, C*H* = CSO_2-*t*), 6.38 (dd, *J* 16.5 and 1 Hz, C*H* = CSO_2-*c*) 6.86 (dd, *J* 16.5 and 10 Hz, SO_2CH=CH_2), 7.57—8.06 (complex, vinylsulphonyl*phenyl*), 8.58 (s, 4-H), 8.65 (d, *J* 1.5 Hz, 9-H) and 8.77 (d, *J* 1.5 Hz, 7-*H*); $\lambda_{max.}$(H_2O) 278 (ε = 23 650) and 428 nm (11 750).

3,3:20,20-*Bisethylenedioxypregn*-5-*en*-11α-*ol* (**4**).—This was prepared according to a published procedure[24] and formed plates from ethyl acetate–hexane, m.p. 214—216 °C (lit.,[24] 214—217 °C); $\nu_{max.}$ 3 600—3 200s, 1 670w, 1 105s, 1 090s, 1 055s, 975m, 960m, 885m, and 870m cm^{-1}; δ 0.80 (3 H, s, 18-H_3), 1.18 (3 H, s, 19-H_3), 1.29 (3 H, s, 21-H_3), 3.94 (5 H, m, OCH_2CH_2O and 11β-H), and 5.39 (1 H, d, *J* 4 Hz, 6-H).

3,3:20,20-*Bisethylenedioxy*-11α-(4-*t*-*butoxycarbonylaminobutyryloxy*)*pregn*-5-*ene* (**5**).—Di-isopropylcarbodi-imide (0.22 ml, 1.44 mmol) and 4-dimethylaminopyridine (17.5 mg, 0.14 mmol) were added to a stirred solution of 3,3:20,20-bisethylenedioxypregn-5-en-11α-ol (**4**) (0.125 g, 0.3 mmol) and 4-t-butoxycarbonylaminobutyric acid (see below) (0.147 g, 0.72 mmol) in dichloromethane (16.5 ml) and the mixture was stirred at room temperature in the dark. After 24 h the solvent was removed under reduced pressure to leave a gum. Excess of di-isopropylcarbodi-imide was removed by repeated addition and evaporation of dichloromethane (3 × 5 ml) and then at 1.0 mmHg. The residue was then dissolved in ethyl acetate (25 ml) and the solution was washed with aqueous $NaHCO_3$ (4 × 5 ml of a solution made from 4 ml of saturated $NaHCO_3$ diluted to 20 ml with water), water (2 × 5 ml), and saturated brine (5 ml), dried ($MgSO_4$), and evaporated under reduced pressure to give a gum (0.356 g). The crude product was filtered through a column of Kieselgel 60 (10 g, particle size 0.2—0.5 mm) using a mobile phase of ethyl acetate–hexane (2:1) and the recovered material (0.324 g) was purified by preparative h.p.l.c. using a mobile phase of ethyl acetate–hexane (1:1). Two products were obtained. The less polar product was the *title compound* (**5**) (0.130 g, 72%), which formed colourless needles from ethyl acetate–hexane, m.p. 158.5—160 °C; $\nu_{max.}$ 3 700—3 100s, 3 480m, 1 715s, 1 695s, 1 265m, 1 245m, 1 200m, 1 170m, 1 090m, and 1 080m cm^{-1}; δ 0.84 (3 H, s, 18-H_3), 1.11 (3 H, s, 19-H_3), 1.26 (3 H, s, 21-H_3), 1.44 (9 H, s, CMe_3), 2.0—2.68 (2 H, complex, includes $HNCH_2CH_2CH_2CO_2$), 3.13 (2 H, q, *J* 6 Hz, $HNCH_2$), 3.94 (4 H, br s, OCH_2CH_2O), 4.4—4.65 (1 H, br, NH), and 5.16—5.48 (2 H, m, 6-H and 11β-H) (Found: C, 67.6; H, 9.0; N, 2.3. $C_{34}H_{53}NO_8$ requires C, 67.6; H, 8.85; N, 2.3%). The second product was the urea derivative (**6**) (90.0 mg), a colourless wax; $\nu_{max.}$ 3 370s, 3 330s, 1 680s, 1 655s, 1 520s, 1 365s, 1 265s, 1 170s, 1 015m, and 640m cm^{-1}; δ 1.22 (6 H, d, *J* 4.5 Hz, Me_2CH), 1.38 (6 H, d, *J* 4.5 Hz, Me_2CH), 1.47 (9 H, s, Me_3C), 1.86 (2 H, m, *J* 5 Hz, $HNCH_2CH_2CH_2CO$), 2.52 (2 H, t, *J* 5 Hz, CH_2CO), 3.20 (2 H, q, *J* 5 Hz, $HNCH_2$), 3.99 (1 H, m, *J* 4.5 Hz Me_2CHNH), 4.36 (1 H, m, *J* 4.5 Hz, Me_2CHN), 4.80 (m, NH), and 7.44 (m, NH).

11α-(4-*t*-*Butoxycarbonylaminobutyryloxy*)*pregn*-4-*ene*-3,20-*dione* (**7b**).—*Method A*. A solution of the bisethylene acetal (**5**) (12.1 mg, 0.02 mmol) and trifluoroacetic acid (93 μl, 1.2 mmol) in tetrahydrofuran (500 μl) and water (50 μl) was stirred at room temperature in the dark for 27 h. The mixture was then diluted with water (10 ml) and rapidly stirred whilst slowly treated with aqueous $NaHCO_3$ (1.0M solution; 1.6 ml, 1.6 mmol). The aqueous solution was then extracted with dichloromethane (1 × 10 ml and 2 × 5 ml) and the combined organic fractions were washed with aqueous $NaHCO_3$ (1.0M; 5 ml), water (2 × 5 ml), and saturated brine (5 ml), dried ($MgSO_4$), and evaporated under reduced pressure to give a white solid (10.2 mg) which was purified by preparative h.p.l.c. using a mobile phase of ethyl acetate–hexane (1:1); two compounds were isolated. The less polar compound was unchanged starting material (**5**) (0.8 mg). The second compound was the title compound (**7b**), a glass (9.2 mg, 90%); $\nu_{max.}$ 3 700—3 150m, 1 725—1 660br, s, 1 615w, 1 370m, 1 250m, and 1 170s cm^{-1}; $\nu_{max.}$($CHCl_3$), 3 450m, 1 725—1 685br s, 1 665s, 1 615w, and 1 260s cm^{-1}; δ 0.74 (3 H, s, 18-H_3), 1.26 (3 H, s, 19-H_3), 1.45 (9 H, s, Me_3C), 2.12 (3 H, s, 21-H_3), 2.18—2.70 (2 H, complex, includes $HNCH_2CH_2CH_2CO_2$), 3.18 (2 H, q, *J* 6 Hz, $HNCH_2$), 4.4—4.65 (1 H, br, NH), 5.27 (1 H, td, 11β-H), and 5.76 (1 H, s, 4-H) (Found: C, 72.4; H, 8.9; N, 3.3. $C_{25}H_{37}NO_4$ requires C, 72.25; H, 9.0, N, 3.4%).

Method B. A solution of the bisethylene acetal (**5**) (12.1 mg, 0.02 mmol) and trifluoroacetic acid (39 μl, 0.5 mmol) in tetrahydrofuran (500 μl) and water (50 μl) was stirred at room temperature in the dark. After 17 h more trifluoroacetic acid (31 μl, 0.4 mmol) and glacial acetic acid (100 μl, 1.75 mmol) were added and the mixture was stirred for a further 4 h. The mixture was then diluted with water (10 ml) and rapidly stirred whilst slowly treated with aqueous $NaHCO_3$ (1.0M solution; 3.0 ml, 3.0 mmol). The aqueous solution was then extracted with dichloromethane (2 × 10 ml) and the combined organic fractions were washed with aqueous $NaHCO_3$ (1.0M solution; 5 ml), water (2 × 5 ml), and saturated brine (5 ml), dried ($MgSO_4$), and evaporated under reduced pressure to give the title compound (**7b**) as a glass (10.2 mg).

Dilithium 6-*Amino*-2-{m-[3-(3,20-*dioxopregn*-4-*en*-11α-*yloxycarbonyl*)*propylaminoethylsulphonyl*]*phenyl*}-1,3-*dioxobenzo*[de]*isoquinoline*-5,8-*disulphonate* (**8**).—A solution of 3,3:20,20-bisethylenedioxy-11α-(4-t-butoxycarbonylaminobutyryloxy)-pregn-5-ene (**5**) (7.4 mg, 0.012 mmol) and trifluoroacetic acid (185 μl, 2.4 mmol) in dioxane (265 μl) and water (80 μl) was stirred at room temperature in the dark for 20 h and then at 60 °C (oil-bath) for 1.25 h. The mixture was then diluted with water (5 ml), cooled, and rapidly stirred whilst slowly treated with aqueous NH_4HCO_3 (1.0M solution; 3.6 ml, 3.6 mmol). The aqueous solution was extracted with dichloromethane (1 × 10 ml and 3 × 5 ml) and the combined organic fractions were washed with water (5 ml), and saturated brine (2 × 5 ml), dried ($MgSO_4$), and evaporated under reduced pressure to give 11α-

(4-*aminobutyryloxy*)*pregn*-4-*ene*-3,20-*dione* (**7a**) (3.3 mg, 67%, 0.008 mmol). This was dissolved immediately in dioxane (2 ml) and water (1 ml). Lucifer Yellow VS (**1**) (4.4 mg, 0.008 mmol) and triethylamine (4 μl, 0.028 mmol) were added to the mixture which was then stirred at room temperature in the dark. After 74 h the mixture was diluted with water (20 ml) and the green fluorescent aqueous solution was extracted with dichloromethane (2 × 4 ml); the aqueous solution was then freeze-dried to give a pale yellow solid (7.5 mg). This solid was dissolved in water (2 ml) and the solution was washed through a column of Dowex 50W-X8 (5 cm × 1 cm i.d., 4 ml volume) prepared in the lithium form. Fractions of the eluate containing product were combined and the solution was freeze-dried to give the *title compound* (**8**) as a pale yellow solid (7.3 mg, 94%); $\nu_{max.}$ 3 700—3 000s, 1 730—1 680br, m, 1 645s, 1 580m, 1 380m, 1 250—1 170br, 1 195s, 1 140m, 1 050m, and 810w cm^{-1}; δ[$(CD_3)_2SO$—D_2O] 1.12 (6 H, br, s, 18-H_3 and 19-H_3), 2.10 (3 H, s, 21-H_3), 2.26—2.6 (2 H, complex, includes $HNCH_2CH_2CH_2CO_2$), 3.6—4.4 (7 H, m, CH_2NHCH_2, SO_2CH_2 and NH), 5.2—5.8 (1 H, m, 11β-H), 6.18 (1 H, s, 4-H), and 7.62—8.6 (7 H, complex m, ArH); $\lambda_{max.}$(H_2O) 229sh (ε 31 420), 257 (19 730), 279.5 (21 550), and 432 nm (10 390); $\lambda_{max.}$(EtOH) 227sh (ε 26 210), 255 (14 140), 279 (17 250), and 432 nm (7 930) (Found: C, 56.1; H, 4.9; Li, 1.2; N, 4.2; S, 10.1. $C_{45}H_{49}Li_2N_3S_3O_{14}$ requires C, 55.95; H, 5.1; Li, 1.4; N, 4.35; S, 10.0%); *m*/*z* 966 ($[M + H]^+$, 5%), 587 (14%), 581 (18%), 435 (15%), and 429 (38%).

4-*t*-*Butoxycarbonylaminobutyric Acid*.—BOC-ON,[25] 2-t-butoxycarbonyloxyimino-2-phenylacetonitrile (0.271 g, 1.1 mmol) was added to a stirred solution of 4-aminobutyric acid (0.103 g, 1.0 mmol) and triethylamine (210 μl, 1.5 mmol) in dioxane (1 ml) and water (1 ml) at room temperature. The resulting heterogeneous mixture was stirred at room temperature in the dark. After 1 h all of the solids had dissolved. The mixture was stirred for a further 15 h and was then diluted with water (20 ml) and ethyl acetate (10 ml). The aqueous layer was extracted with more ethyl acetate (10 ml) and then acidified with 2M sulphuric acid (0.3 ml) to produce a white suspension. The mixture was extracted with ethyl acetate (3 × 10 ml), and the combined organic fractions were washed with water (2 × 5 ml), dried ($MgSO_4$), and evaporated under reduced pressure to give the *title compound* as a colourless glass (0.2 g); $\nu_{max.}$ 3 700—2 300br, 3 350s, 2 980s, 2 950s, 1 720—1 680br, s, 1 520m, 1 280m, 1 270m, 1 255m, and 1 170s; δ 1.52 (3 H, s, Me_3C), 1.94 (2 H, m, *J* 7 Hz, $HNCH_2CH_2CH_2CO_2H$), 2.47 (2 H, t, *J* 7 Hz, CH_2-CH_2CO_2H), 3.21 (2 H, t, *J* 7 Hz, $HNCH_2CH_2$), 4.6—5.2 (br, NH), and 10.26 (1 H, s, CO_2H).

Acknowledgements

We thank the M.R.C. for a grant (to D. N. K.) for the Steroid Reference collection, and Professor V. H. T. James, Department of Chemical Pathology, St. Mary's Hospital Medical School, London, for providing facilities for fluorescence measurements.

References

1 'Radioimmunoassay of Steroid Hormones,' ed. D. Gupta, 2nd Edn., Verlag Chemie, Weinheim, 1980.
2 E. Soini and I. Hemmila, *Clin. Chem.*, 1979, **25**, 353.
3 D. S. Smith, M. M. H. Al-Hakiem, and J. Landon, *Ann. Clin. Biochem.*, 1981, **18**, 253.
4 M. M. Martin and L. Lindqvist, *J. Lumin.*, 1975, **10**, 381.
5 J. R. Falck, M. Krieger, J. L. Goldstein, and M. S. Brown, *J. Am. Chem. Soc.*, 1981, **103**, 7396.
6 C. Parini, M. A. Bacigalupo, S. Colombi, L. Ferrara, F. Franceschetti, and R. Saita, *Steroids*, 1985, **46**, 903.
7 M. A. Bacigalupo, A. Ius, G. Meroni, R. Saita, and C. Parini, *J. Steroid Biochem.*, 1983, **19**, 1661.
8 K. Pettersson, H. Siitari, I. Hemmila, E. Soini, T. Lovgren, V. Hanninen, P. Tanner, and U.-H. Stenman, *Clin. Chem.*, 1983, **29**, 60.
9 E. Soini and H. Kojola, *Clin. Chem.*, 1983, **29**, 65.
10 U.-H. Stenman, H. Alfthan, L. Myllynen, and M. Seppala, *The Lancet*, 1983, **ii**, 647.
11 H. Siitari, I. Hemmila, E. Soini, T. Lovgren, and V. Koistinen, *Nature*, 1983, **301**, 258.
12 G.-y. Adachi, K. Tomokiyo, K. Sorita, and J. Shiokawa, *J. Chem. Soc., Chem. Commun.*, 1980, 914.
13 M. P. Bailey, B. F. Rocks, and C. Riley, *Ann. Clin. Biochem.*, 1983, **20**, 213.
14 A. Tsuji, *Bunseki Kagaku*, 1982, **31**, E333.
15 B. L. Allman, F. Short, and V. H. T. James, *Clin. Chem.*, 1981, **27**, 1176.
16 W. W. Stewart, *J. Am. Chem. Soc.*, 1981, **103**, 7615.
17 W. W. Stewart, *Cell*, 1978, **14**, 741.
18 W. W. Stewart, *Nature*, 1981, **292**, 17.
19 L. F. Fieser and M. Fieser, 'Reagents for Organic Synthesis,' Wiley, New York, 1967, vol. 1, pp. 231—236.
20 K. Holmberg and B. Hansen, *Acta Chem. Scand., Ser. B*, 1979, **33**, 410.
21 T. L. Kirley and H. B. Halsall, *J. Steroid Biochem.*, 1982, **16**, 133.
22 J. G. Liehr, C. F. Beckner, A. M. Ballatore, and R. M. Caprioli, *Steroids*, 1982, **39**, 599.
23 D. D. Perrin, W. L. F. Armarego, and D. R. Perrin, 'Purification of Laboratory Chemicals,' Pergamon, London, 1966.
24 G. Cooley, B. Ellis, D. N. Kirk, and V. Petrow, *J. Chem. Soc.*, 1957, 4112.
25 M. Itoh, D. Hagiwara, and T. Kamiya, *Tetrahedron Lett.*, 1975, 4393.

Received 12*th January* 1988; *Paper* 8/00116B