Synthesis of haptens and their protein conjugates for immunological determination of nitrate esters and nitramines

G. Michael Blackburn,*[a] Ian G. Beadham,[a] Harry Adams,[a] Alistair P. Hutchinson[b] and Stephen Nicklin[b]

[a] *Krebs Institute for Biomolecular Research, Department of Chemistry, University of Sheffield, Brookhill, Sheffield, UK S3 7HF*
[b] *Defence Evaluation and Research Agency, Fort Halstead, Kent, UK TM14 7BP*

Received (in Cambridge, UK) 24th August 1999, Accepted 18th November 1999

Isosteric, isopolar analogues of the nitrate ester pentaerythritol tetranitrate (PETN) and of the cyclic nitramine *sym*-cyclotrimethylene trinitramine (RDX) having a spacer arm have been linked to carrier proteins to provide immunologically active peptide conjugates. X-Ray structures of RDX, RDX analogue **10**, and hapten **14** are compared to establish their conformational relationships.

Organic nitrate esters such as pentaerythritol tetranitrate (PETN, **1**), nitroglycerine (NG, **2**) and isosorbide dinitrate **3** have been used in the treatment of angina for over a hundred years (Chart 1).[1] However, it has only recently emerged that

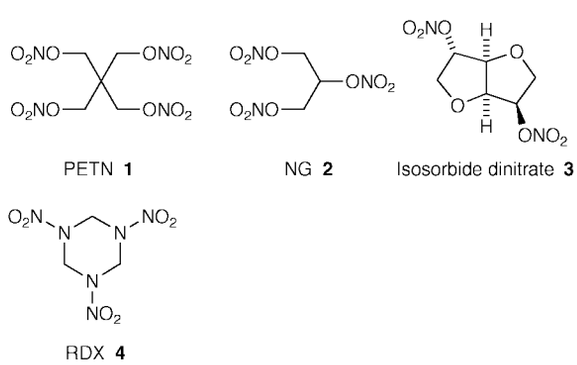


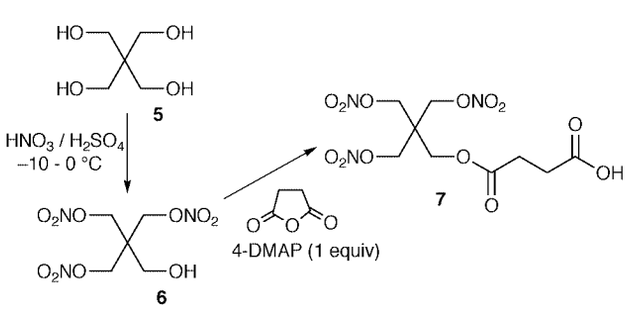
Chart 1 Nitrate esters and nitramines with biological activity.

organic nitrates such as these are in fact prodrugs which activate guanylate cyclase by releasing nitric oxide *in vivo*. The active therapeutic agent is now believed to be either nitric oxide itself, or an *S*-nitroso thiol (thionitrile).[2] As with other medicines, side-effects can occur when the prescribed dose of an organic nitrate is exceeded. Overdoses of nitroglycerine can cause headaches, and fatalities have occurred when small doses of NG have been taken with alcohol or the painkillers codeine and morphine.[3] The cyclic nitramine, *sym*-cyclotrimethylene trinitramine (RDX, **4**) (Chart 1) is a related molecule with potential to release oxides of nitrogen (NO_x) *in vivo* which was originally patented as a medicine[4] although subsequently it has also been found to be a viable rodenticide.[5] NG, PETN, and RDX also possess explosive properties and as such have been widely used by the military for many years in shells and demolition charges.[6,7] Indeed, one of the major environmental problems facing governments world-wide is the detection and remediation of explosives contamination arising from training, war, and/or manufacture of such materials. Thus, the multifunctional nature and wide application of these nitrated alcohols and amines makes them interesting targets for monitoring and detection, for both medical and environmental purposes.

Results and discussion

In order to develop a test for the target organic nitrates and nitramines, we have focused on immunological methods of detection. The usefulness of *immunoassays* stems from the exquisitely selective binding possible between an antibody and its antigen.[8] To elicit antibodies to a molecule with molecular mass less than one kilodalton, it is necessary to synthesise an isosteric, isopolar analogue of the molecule of interest which has a flexible arm through which it can be linked to an immunogenic carrier protein and so used as a hapten to stimulate the mammalian immune system.[8b,9]

For the target PETN **1**, we chose pentaerythritol trinitrate (Petrin, **6**) as the hapten. This retains three of the four nitrate esters and most of the structural and conformational features of PETN while it can be linked to carrier proteins *via* extension through the free hydroxy group. Petrin was synthesised using the method of Camp[10] in which commercially available pentaerythritol **5** is treated with a mixture of conc. nitric and sulfuric acid (1 : 1, *v*/*v*) and urea to give Petrin in 35% yield with PETN as a side-product in approximately equal amount. Petrin was acylated with succinic anhydride to append a terminal carboxy group for amide linkage to the multiple lysine residues of the carrier proteins BSA (bovine serum albumin) and KLH (keyhole limpet haemocyanin) (Fig. 2). Trinitropentaerythritol hemisuccinate **7** was prepared in 56% yield by using 4-DMAP (1 mole equivalent) as both base and nucleophilic catalyst (Scheme 1). We note that our melting point (mp) of 139 °C for **7** is significantly higher than that (88–99 °C) reported by Marans.[11]



Scheme 1 Synthesis of an analogue of PETN.

The structure of RDX **4** offers little opportunity for direct attachment of a linker without serious loss of isosteric and isopolar relationship to the parent molecule. The conformation of RDX has been well characterised by X-ray diffraction, having a chair ring with two pyramidal nitrogens carrying axial N-nitro groups and the third, more nearly trigonal, nitrogen has its nitro group lying a little above the median plane of the ring (Fig. 1A).[12] The two axial nitro groups and the ring are bisected

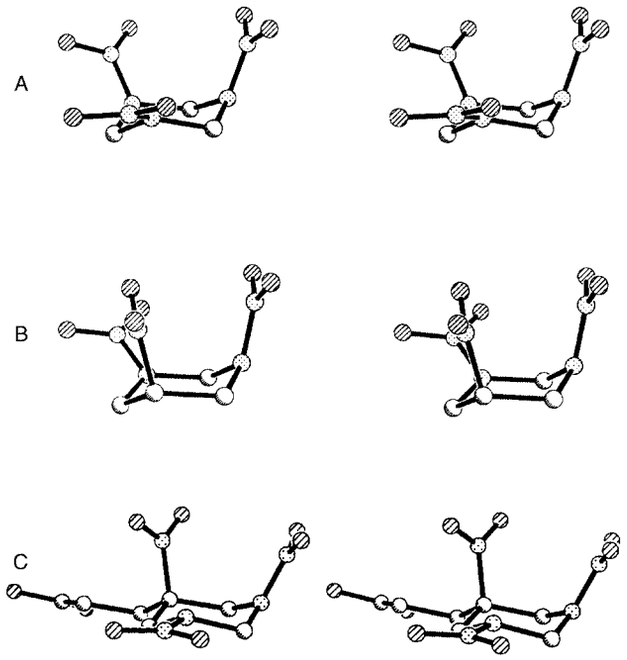
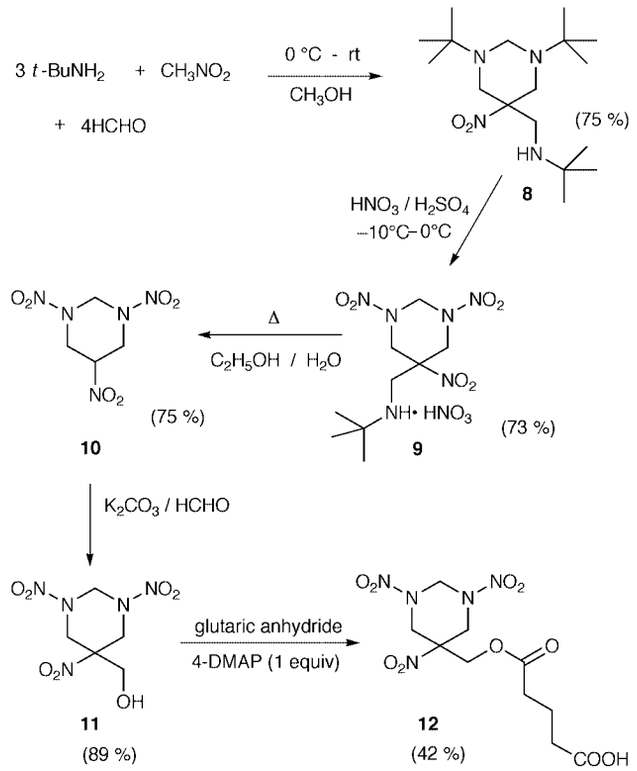


Fig. 1 (**A**) Solid-state structure of RDX **4** (coordinates from ref. 12), (**B**) solid-state structure of RDX analogue **10**, and (**C**) solid-state structure of hapten **14**. (3-D pairs for parallel viewing). Plain circles = C-atoms, speckled circles = N-atoms, hatched circles = O-atoms.



Scheme 2 Synthesis of RDX analogue **10** and its conversion into a hapten.

by a pseudo-mirror plane. We therefore selected the cyclic nitramine 1,3,5-trinitrohexahydropyrimidine (TNP, **10**) as a mono-deaza analogue of RDX which can be linked *via* a spacer arm joined at C-5 to a carrier protein and might still retain most of these structural and conformational features.

TNP **10** was first prepared by Ritter and Licht[13] in 1985 and its synthesis proved uneventful with all three steps proceeding in good yield (Scheme 2). TNP was condensed with formaldehyde[13] using aq. potassium carbonate. Acylation of the resulting product **11** with glutaric anhydride and 4-DMAP afforded the novel analogue of RDX **12** as a hapten ready for linkage to a carrier protein.

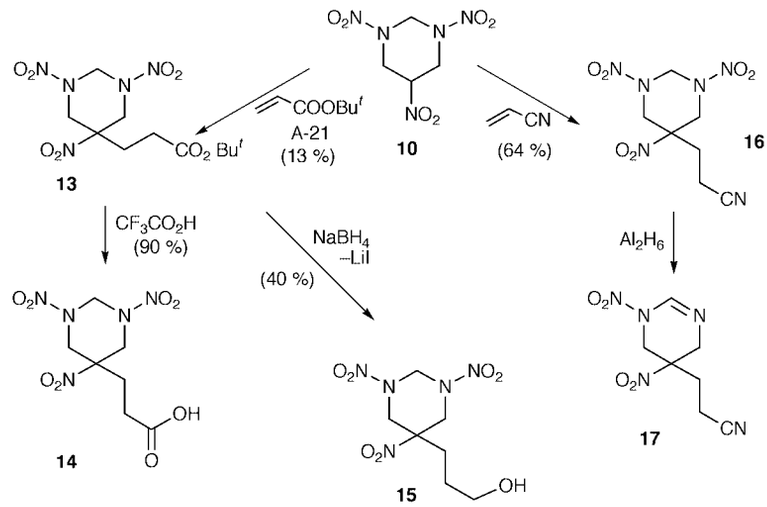
An alternative linker designed to exhibit greater resistance to hydrolysis *in vivo* was attached to the TNP core by first treating *tert*-butyl acrylate with TNP in the presence[14] of the weakly basic anionic resin Amberlyst® A-21. This resulted in formation of the *tert*-butyl ester **13**. The low yield of this step (13%) probably reflects the slowness of 1,4-addition to *tert*-butyl acrylate, competing with base-promoted decomposition of TNP. Indeed, even marginally stronger bases such as pyridine (pK_a 5.2) induce complete decomposition of TNP without 1,4-addition. The succeeding step, cleavage of the *tert*-butyl group, took place smoothly in the presence of excess of TFA, affording the required carboxylic acid **14** in 90% yield (Scheme 3). This *tert*-butyl ester **13** was also reduced to the hydroxypropyl derivative **15** using sodium borohydride–lithium iodide[15] ($LiEt_3BH$ was also used to effect this reduction). While this product was not further developed for immunisation purposes, it was available as a back-up hapten to be deployed with a longer spacer should that have proved immunologically desirable.[16]

To explore further the reactivity of **10** under Michael additions, it was treated with acrylonitrile and Amberlyst A-21 to give 5-(2-cyanoethyl)-1,3,5-trinitrohexahydropyrimidine **16** in good yield. However, attempts to hydrolyse the cyano function of this product led to general decomposition. Brown and Yoon[17] have described the use of aluminium hydride, Al_2H_6, for the selective reduction of aliphatic nitriles. However, on treatment of **16** with this reagent the only product obtained was nitroamidine **17**, formally derived by elimination of HNO_2. This result is consistent with Lamberton's observations on the sensitivity of N-nitro groups towards reducing agents.[18]

X-Ray-quality crystals of RDX analogue **10** and of RDX-analogue hapten **14** were isolated by evaporation of solutions in ethanol and methanol, respectively, and the resulting X-ray structures determined. The structure of RDX analogue **10** shows a chair conformation. The nitro groups on tetrahedral C-5 and pyramidal N-1 are axial and that on the trigonal N-3 lies above the average plane of the ring (Fig. 1B). The structure of hapten **14** shows this species also has a chair conformation, with the three-carbon side-chain at C-5 in an equatorial position. The two nitro groups at pyramidal N-1 and tetrahedral C-5 are axial and the nitro group at the more nearly trigonal N-3 is quasi-equatorial (Fig. 1C). These structures clearly show that the attachment of the three-carbon chain to C-5 of analogue **10** to give hapten **14** has a major beneficial effect on their structural relationship to RDX **4**. This is clearly seen in the superposition of the structure of RDX on those of **10** and **14** (Fig. 2A,B).

Of three modes for superposing the six ring atoms of **4** on those of **14**, all gave a high quality of fit for the six ring atoms (RMS deviation for the six ring atoms ≤ 0.074 Å). The optimum superposition (RMS deviation 0.0473 Å) was analysed in some detail (Fig. 2A) and shows a very good conformational fit for the three nitro groups. For the axial nitro groups at N-1, the two nitrogens are 0.124 Å with O–O separations of 0.045 and 0.378 Å. The nitro groups at N-3 are approximately planar to their rings, with an N–N separation of 0.410 Å and O–O separations of 0.441 and 0.677 Å. The two axial nitro groups at C-5 of **14** and N-5 of **4** show good superposition of their nitrogen atoms (N–N separation 0.425 Å) with O–O separations of 0.413 and 0.960 Å (Fig. 2A).

In contrast, the superposition of structures of RDX **4** and analogue **10** show major discrepancies between the orientation of the nitro groups (Fig. 2B). Of three modes for superposing the six ring atoms of **4** on those of **10**, all gave a good quality of fit with the best having RMS deviation for the six ring atoms of 0.0686 Å. This overlay was analysed in detail (Fig. 2B) and shows a modest conformational fit for the three nitro groups. The axial nitro groups at N-1 show a good fit with N–N separ-



Scheme 3 Attachment of alternative linkers to RDX analogue **10**.

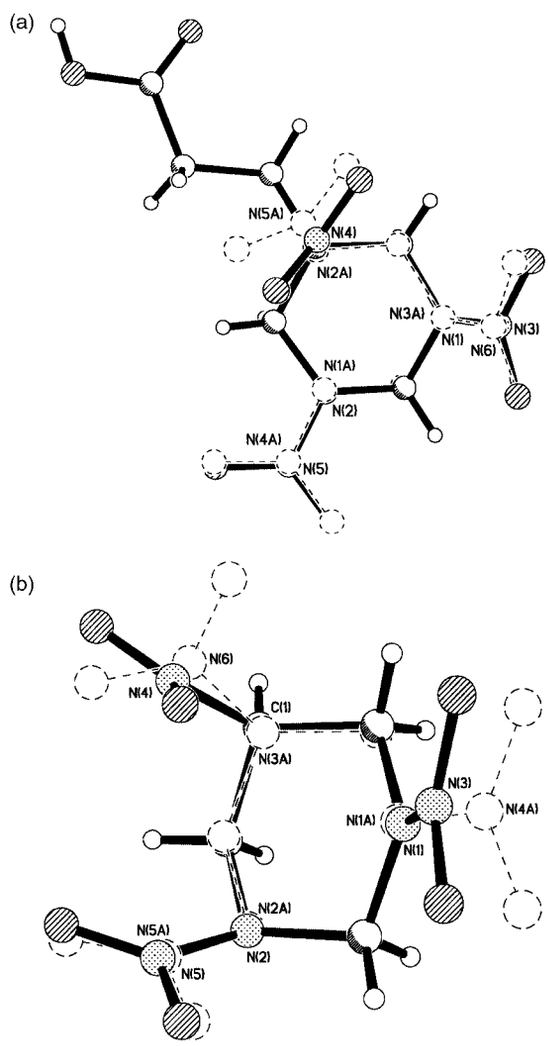
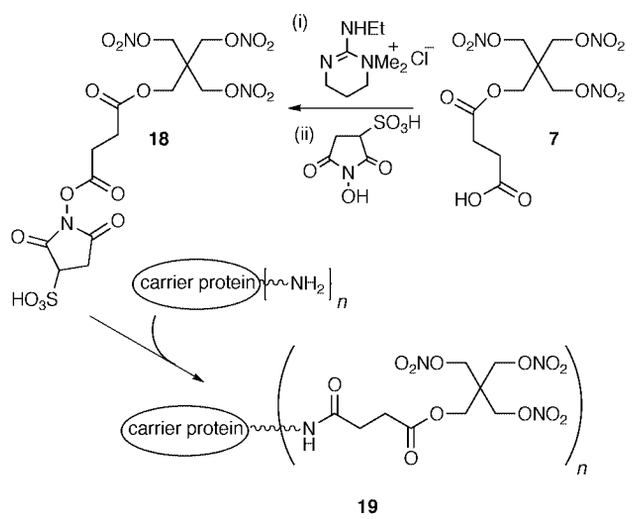


Fig. 2 Superposition of crystal structures for (A) RDX **4** (dashed outline) and hapten **14** (bold outline) and (B) RDX **4** (dashed outline) and analogue **10** (bold outline) based on optimised fit for the six ring atoms.

ation of 0.098 Å and with O–O separations of 0.103 and 0.225 Å. N-3 in analogue **10** is the most nearly trigonal of all ring nitrogens and the N–N separation of these two nitro groups is 0.579 Å with O–O separations of 0.754 and 0.881 Å. The two axial nitro groups at C-5 of **10** and N-5 of **4** show fair superposition of their nitrogen atoms (N–N separation 0.355 Å) but with larger O–O separations of 0.715 and 1.708 Å. This arises from a change in orientation of the C^5-nitro group in **10** relative to the N^5-nitro group in **4** to a radial from a tangential conformation relative to the ring.

These data fully endorse our choice of **14** as an isopolar and isosteric analogue of RDX **4**. While we were unable to generate crystals of **12** suitable for diffraction purposes, it seems likely from an analysis of solution-phase ^{1}H NMR data that there is sufficient conformational similarity between these two RDX analogues to justify the use of **12** as a hapten for the generation of antibodies with high affinity for RDX. (We appreciate that the solid-state conformations of analogue **14** and RDX **4** are not necessarily the same as those in aqueous solution, in which recognition by the immune system must take place).

Analogues **7**, **12** and **14** were attached to the carrier proteins BSA, KLH, and hen ovalbumin (OVA) *via* water-soluble *N*-hydroxysulfosuccinimido esters (generated with 1-[3-(dimethylamino)propyl]-3-ethyl carbodiimide hydrochloride, EDC, and *N*-hydroxysulfosuccinimide, sulfo-NHS, in an aqueous solution containing the carrier protein).[19] This process is exemplified by *in situ* formation of active ester **18** using PETN analogue **7** (Scheme 4).



Scheme 4 Formation of a water-soluble activated ester and its linking to a carrier protein to form immunogenic hapten–protein conjugates.

The active ester **18** was used to acylate residues (especially lysines) which have nucleophilic side-chains, on the surface of the carrier proteins. In this way, multiple copies of the hapten

were linked to the surface of the carrier protein to form an immunogenic hapten–protein conjugate **19** (Scheme 4).

Initially, monoclonal antibodies which cross-reacted with PETN were isolated by Nicklin and Hutchinson[20] *via* protein conjugates of **7**. Using a similar immunisation protocol, we derived immunogenic conjugates using, in turn, the PETN analogue **7** and active esters derived from RDX-analogue haptens **12** and **14**. Following extensive immunisation studies using these conjugates, monoclonal antibodies cross-reacting to RDX were isolated.

Preliminary competition enzyme-linked immunosorbent assay (ELISA) binding studies have shown that the affinities of monoclonal antibodies raised against these haptens lie within the range required for immunoassay (K_d 10^{-6}–10^{-9} M) and they have the capacity to detect both PETN and RDX in explosives-contaminated soils and waste streams. The utility of these reagents for monitoring RDX and PETN in the clinical environment remains to be determined.

Experimental

Mps were measured on a Reichert hot-stage micro-melting point apparatus and are uncorrected. Proton NMR were recorded on a Bruker AC-250 spectrometer at 250 MHz. Chemical shifts are reported in the δ scale in ppm, using the solvent as an internal standard. *J*-values are in Hz. Carbon NMR spectra were recorded on a Bruker AC-250 at 62.9 MHz. Chemical shifts are quoted in parts per million. IR spectra were recorded on a Perkin-Elmer 457 grating infra-red spectrometer using either KBr discs or thin films. UV spectra were recorded on a Philips PU 8720 spectrophotometer for solution in ethanol. Elemental analyses were determined on a Perkin-Elmer 2400 CHN elemental analyser to within ±0.4% of the theoretical values unless otherwise stated. Electron-impact (EI) and chemical-ionisation (CI) mass spectra were recorded on either a Kratos MS25 or a Fisons Instruments Prospec 3000 mass spectrometer. Solvents were purified as follows. THF and diethyl ether (hereafter abbreviated to ether) were heated under reflux over sodium and benzophenone and distilled when the solution turned purple. DCM was heated under reflux over calcium hydride and then distilled. Acetonitrile was purchased HPLC-grade anhydrous solvent. BSA, KLH, OVA and sulfo-NHS were obtained from Pierce & Warriner, Chester, UK. R_f-values were obtained using DC-Alufolien TLC plates from Merck. Traces were detected using either UV, alkaline potassium permanganate or 2,4-dinitrophenol (2,4-DNP). Column chromatography was carried out using Kieselgel 60, 230–400.

Pentaerythritol trinitrate 6

Pentaerythritol **5** (24 g, 0.18 mol) was added to conc. nitric acid (88 ml) and urea (0.3 g, 5.0 mmol), the mixture cooled to −11 °C in a salt–ice-bath, and stirred for 2 min. Sulfuric acid (98%; 90 ml) was then added dropwise, maintaining a reaction temperature below 7 °C. After stirring of the mixture for 2 h, the white solid (pentaerythritol tetranitrate **1**) which had formed was filtered off, precipitated from a saturated acetone solution by addition of aq. ethanol and filtered off again. The combined filtrates were added to water (350 ml) and stored for 16 h to precipitate further pentaerythritol tetranitrate. This was filtered off, the aqueous layer extracted with chloroform (200 ml), and the combined organic layers dried over magnesium sulfate. The solvent was evaporated at water-pump pressure to afford pentaerythritol trinitrate **6** as a pale yellow oil (17.0 g, 35%), ν_{max}(neat)/cm^{-1} 3600, 3400 (OH), 1650 (NO_2 asym), 1470, 1375 (NO_2 sym), 1275, 1055; δ_H ($CDCl_3$) 3.80 (6 H, s, 3 × CH_2ONO_2), 4.60 (2 H, s, CH_2O).

Pentaerythritol trinitrate hemisuccinate 7

Pentaerythritol trinitrate (2.0 g, 4.6 mmol), succinic anhydride (0.72 g, 4.9 mmol) and 4-(dimethylamino)pyridine (4-DMAP) (0.62 g, 5.1 mmol) were stirred in freshly distilled THF (10 ml) for 72 h at rt. The solvent was then removed *in vacuo* below 10 °C, the residue dissolved in ethyl acetate (100 ml) and the solution washed with hydrochloric acid (2 × 150 ml; 0.1 M). The organic extracts were dried over magnesium sulfate and concentrated to afford the title compound **7** as an off-white solid (2.9 g, 56%), mp 139 °C (lit.,[11] 88–99 °C) [Found: C, 29.58; H, 3.42; N, 10.97. Calc. for $C_9H_{13}N_3O_{13}$ (371.045): C, 29.12; H, 3.53; N, 11.32%]; λ_{max}/nm 209.4 (ε/dm^3 mol^{-1} cm^{-1} 5773); ν_{max}/(KBr disc)/cm^{-1} 3460 br (CO-H), 1746 (C=O), 1646, 1260, 994; δ_H ($CDCl_3$) 2.60–2.64 (4 H, m, CH_2CH_2), 4.20 (2 H, s, CH_2OCO), 4.50 (6 H, s, 3 × CH_2ONO_2); δ_C ($CDCl_3$) 174.7 (RCOOH), 171.7 (ROCOR), 69.3 (CH_2ONO_2), 61.5 [CH_2O-CO(R)], 42.2 [R$C(CH_2ONO_2)_3$], 28.9, 28.7 (ROCOCH_2CH_2-CO_2H); *m*/*z* (EI) 353 ([M − H_2O], 45%), 308 (16), 250 (42), 219 (40), 205 (100).

1,3-Di-*tert*-butyl-5-(*tert*-butylaminomethyl)-5-nitrohexahydropyrimidine 8

An aqueous solution of formaldehyde (32 ml, 0.40 mol; 37%) was added to a solution of *tert*-butylamine (22 g, 0.30 mol) in methanol (200 ml) and the mixture was stirred at 10 °C for 5 min before addition of nitromethane (5.4 ml, 0.10 mol). After 10–15 min a white solid precipitated. The flask was cooled in ice and the crystals filtered off to afford the title compound **8** (26 g, 75%), mp 104 °C (lit.,[13] 103–104 °C) [Found: C, 61.91; H, 11.08; N, 17.02. Calc. for $C_{17}H_{36}N_4O_2$ (328.28): C, 62.14; H, 11.05; N, 17.06%]; λ_{max}/nm 217.9, 270.5 (ε/dm^3 mol^{-1} cm^{-1} 32561, 375); ν_{max} (KBr disc)/cm^{-1} 1546 (NO_2 asym), 1471, 1442 (NO_2 sym); δ_H ($CDCl_3$) 1.03 [9 H, s, NBu^t (aliphatic)], 1.10 [18 H, s, 2 × NBu^t (heterocyclic)], 2.70 [2 H, d, *J* 12.5, CH_2 (4,6)], 3.00 (1 H, s, NH), 3.23 [1 H, d, *J* 12.5, $CH^{a,b}$ (2)], 3.35 [2 H, d, *J* 12.5, CH_2 (4,6)], 3.50 [2 H, s, CH_2 (7)], 3.55 [1 H, d, *J* 12.5, $CH^{a,b}$ (2)]. δ_C [$(CD_3)_2SO$] 26.5 [CH_3 (ring N)], 29.1 [CH_3 (aliphatic N)], 48.8 (quat. C aliphatic Bu^t), 50.1 [quat. CBu^t (ring)], 50.7 [CH_2 (7)], 53.3 [CH_2 (4,6)], 64.2 [CH_2 (2)], 89.8 [C (5)]; *m*/*z* (EI) 272 [M + H − Bu^t]$^+$ (13%), 256 [M − NHBu^t]$^+$ (16), 242 [M − CH_2NHBu^t]$^+$ (14).

5-(*tert*-Butylaminomethyl)-1,3,5-trinitrohexahydropyrimidine 9

Compound **8** (2.47 g, 7.5 mmol) was added slowly, with stirring, to 98% sulfuric acid (25 ml, 0.5 mol) over 2 h, maintaining the temperature at less than 10 °C until an orange solution formed. Conc. nitric acid (specific gravity 1.42, analytical grade; 6.25 ml, 0.15 mol) was then added dropwise and, after a further 20 min of stirring at 0 °C, the reaction was quenched by pouring onto an ice–water slurry. A cream-white solid precipitated out and was filtered off, and washed with ether and a little acetone to afford the nitrate salt **9** of the title compound as an off-white solid (2.0 g, 73%), mp 154 °C (decomp.) [lit.,[13] 149–152 °C (crude), 175 °C (pure)] (Found: C, 29.3; H, 5.2; N, 26.6. Calc. for $C_9H_{19}N_7O_9$: C, 29.3; H, 5.1; N, 26.3%); λ_{max} 233.9 nm (ε/dm^3 mol^{-1} cm^{-1} 15631) (as nitrate salt); ν_{max} (KBr disc)/cm^{-1} 3400 (NH), 2750–3100, 1560–1575, 1550 (NO_2 asym), 1375 (NO_2 sym), 1250–1350 (as nitrate salt); δ_H [$(CD_3)_2SO$] 1.50 (9 H, s, Bu^t), 3.80 [2 H, d, *J* 12.5, CH_2 (7)], 4.25 [2 H, d, *J* 12.5, CH_2 (4,6)], 5.10 [1 H, d, *J* 12.5, $CH^{a,b}$ (2)], 5.45 [2 H, d, *J* 12.5, CH_2 (4,6)], 7.25 [1 H, d, *J* 12.5, $CH^{a,b}$ (2)], 8.80 [2 H, br s, NH_2); δ_C [$(CD_3)_2SO$] 85.6 [CH_2 (2)], 63.2 (CNO_2), 60.2 [$C(CH_3)_3$], 50.9 [CH_2 (4,6)], 28.9 (CH_3); *m*/*z* (EI) 307 ([M + H]$^+$, 0.5%).

1,3,5-Trinitrohexahydropyrimidine 10

Compound **9** (as nitrate salt) (6.32 g, 15 mmol) was refluxed in aq. ethanol (30 ml; 81%) for 20 min to afford the title compound **10**, which precipitated out as sparkling plates on cooling (2.5 g, 75%), mp 141 °C (lit.,[13] 142–144 °C) (Found: C, 21.77; H, 3.18; N, 31.78. Calc. for $C_4H_7N_5O_6$: C, 21.72; H, 3.19; N,

31.68%); λ_{max} 236.8 nm (ε/dm^3 mol^{-1} cm^{-1} 9917); ν_{max} (KBr disc)/cm^{-1} 1568 (NNO_2 asym), 1532 (NO_2 asym), 1329 (NO_2 sym), 1297, 1256 (NNO_2 sym); δ_H [$(CD_3)_2CO$] 6.67 [1 H, d, *J* 16.7, CH^{a,b} (2)], 5.60 [1 H, d, *J* 16.7, CH^{a,b} (2)], 5.35 [1 H, quintet, CH (5)], 5.18 [2 H, dd, *J* 4.7, 16.7, CH_2 (4,6)], 4.53 [2 H, dd, *J* 4.7, 16.7, CH_2 (4,6)]; δ_C [$(CD_3)_2CO$] 48.3 [CH_2 (4,6)], 61.3 [CH_2 (2)], 76.2 [CH (5)]; *m*/*z* (EI) 222 ([M + H]$^+$, 16%).

X-Ray crystal data for 10.† $C_4H_7N_5O_6$; M = 221.15, crystallises from ethanol as colourless blocks; crystal dimensions 0.50 × 0.35 × 0.20 mm. Monoclinic, *a* = 12.339(9), *b* = 6.574(4), *c* = 11.163(16) Å, β = 109.86(8)°, *V* = 851.6(15) Å^3, *Z* = 4, D_c = 1.725 Mg m^{-3}, space group $P2_1/c$ ($P2_1/c$ $C_{h'}$ No. 14), Mo-Kα radiation (λ = 0.710 73 Å), μ(Mo-Kα) = 0.161 mm^{-1}, *F*(000) = 456.

Three-dimensional, room temperature X-ray data were collected in the range 3.5° < 2θ < 40° on a Siemens P4 diffractometer by the omega-scan method. Of the 888 reflections measured, all of which were corrected for Lorentz and polarisation effects and for absorption by semi-empirical methods based on symmetry-equivalent and repeated reflections (minimum and maximum transmission coefficients 0.9237 and 0.9685), 696 independent reflections exceeded the significance level $|F|/\sigma(|F|) > 4.0$. The structure was solved by direct methods and refined by full-matrix least-squares methods on F^2. Hydrogen atoms were included in calculated positions and refined in riding mode. Refinement converged at a final *R* = 0.0394 (w*R*2 = 0.1104 for all 803 unique data, 137 parameters, mean and maximum δ/σ 0.000, 0.000), with allowance for the thermal anisotropy of all non-hydrogen atoms. Minimum and maximum final electron density −0.162 and 0.146 e Å^{-3}. A weighting scheme $w = 1/[\sigma^2(F_o^2) + (0.0684*P)^2 + 0.458*P]$ where $P = (F_o^2 + 2*F_c^2)/3$ was used in the latter stages of refinement. Complex scattering factors were taken from the program package[21] SHELXL-97 as implemented on a Viglen 486dx computer.

5-Hydroxymethyl-1,3,5-trinitrohexahydropyrimidine 11

A saturated solution of sodium carbonate (3 ml) in formalin (10 ml, 0.1 mol; 35%) was added to a solution of **10** (11.1 g, 0.05 mol) in ethyl acetate (500 ml). After stirring for 2 h at rt, the ethyl acetate solution was decanted from the solid residue, dried over anhydrous sodium sulfate, and concentrated to afford a viscous oil. Column chromatography (DCM eluent) afforded the alcohol **11** (11.2 g, 89%), mp 142 °C (lit.,[13] 140–142 °C); λ_{max} 238.4, 348.0 (ε/dm^3 mol^{-1} cm^{-1} 0.645, 0.039); ν_{max} (KBr disc)/cm^{-1} 3500, 2800–3100 (OH), 1550 (N–NO_2 asym), 1530 (NO_2 asym), 1375 (NO_2 sym), 1300, 1275 (N–NO_2 sym); δ_H [$(CD_3)_2SO$] 3.90 (2 H, d, *J* 6.4, CH_2O), 4.10 [2 H, d, *J* 15.2, CH_2 (4,6)], 5.15 [1 H, d, *J* 15.2, CH^{a,b} (2,2)], 5.25 [2 H, d, *J* 15.2, CH_2 (4,6)], 5.90 (1 H, t, *J* 6.4, OH), 6.80 [1 H, d, *J* 15.2, CH^{a,b} (2,2)]; δ_C [$(CD_3)_2SO$] 49.9 [CH_2 (7)], 61.2 [CH_2 (4,6)], 64.7 [CH_2 (2)], 88.1 [CNO_2 (5)]; *m*/*z* (EI) 252 ([M + H]$^+$, 2%), 234 ([M − OH]$^+$, 7), 204 ([M − HNO_2]$^+$, 15).

(1,3,5-Trinitrohexahydropyrimidin-5-yl) methylhemiglutarate 12

Glutaric anhydride (0.24 g, 2.1 mmol) was added to a stirred solution of 4-DMAP (0.28 g, 2.5 mmol) and **11** (0.52 g, 2.1 mmol) in acetonitrile (30 ml). After 8 h, the orange-brown solution was worked up by addition of hydrochloric acid (55 ml; 1.1 M) and extraction with ethyl acetate. The organic phase was dried over Na_2SO_4 and filtered, and the solvent was evaporated off to afford the *title compound* **14**. Purification was carried out by repeated precipitation from a minimum of acetonitrile by addition of DCM to give the title compound as a white solid (0.32 g, 42%), mp 151 °C (Found: C, 30.49; H, 3.68; N, 21.07. $C_{10}H_{15}N_5O_{10}$ requires C, 32.90; H, 4.14; N, 19.18%). λ_{max}/nm 238.5 (ε/dm^3 mol^{-1} cm^{-1} 9180); ν_{max} (KBr disc)/cm^{-1} 1756 (C=O acid), 1570 (NNO_2 asym), 1458 (NO_2 asym), 1388 (NO_2 sym), 1345, 1297 (NNO_2 sym); δ_H [$(CD_3)_2SO$] 1.73 [2 H, quintet, *J* 6.3, CH_2 (9)], 2.25 [2 H, t *J* 7.8, CH_2 (8)], 2.40 [2 H, t *J* 7.8, CH_2 (10)], 4.20 [2 H, d, *J* 15.6, CH_2 (4,6)], 4.60 (2 H, s, CH_2O), 5.13 [1 H, d, *J* 15.6, CH^{a,b} (2)], 5.30 [2 H, d, *J* 15.6, CH_2 (4,6)], 6.90 [1 H, d, *J* 15.6, CH^{a,b} (2)], 12.12 (1 H, s, OH); δ_C [$(CD_3)_2SO$] 20.4 [CH_2 (9)], 32.9 [CH_2 (8)], 33.2 [CH_2 (10)], 49.1 [CH_2 (7)], 60.7 [CH_2 (4,6)], 63.8 (2), 84.5 (5), 172.1 (CH_2OCO), 174.5 (CO_2H); *m*/*z* (CI) 366 ([M + H]$^+$, 18%), 348 ([M − OH]$^+$, 31).

5-[2′-(*tert*-Butoxycarbonyl)ethyl]-1,3,5-trinitrohexahydropyrimidine 13

Amberlyst A-21 (18.7 g) and **10** (4.0 g, 18.1 mmol) were stirred in *tert*-butyl acrylate (42 ml) overnight. Resin was removed by filtration and excess of *tert*-butyl acrylate was removed at reduced pressure. Flash column chromatography (ether) afforded the title compound **13** as a white solid (0.8 g, 13%), R_f 0.4 (ether); λ_{max}/nm 203.1 (ε/dm^3 mol^{-1} cm^{-1} 13 473); ν_{max} (KBr disc)/cm^{-1} 1723.4 (C=O), 1552.8 (NNO_2 asym), 1431.4 (NO_2 asym), 1384.1 (NO_2 sym), 1345.0 (NNO_2 sym); δ_H ($CDCl_3$) 1.40 [9 H, s, $(CH_3)_3$], 2.15–2.35 [4 H, m, CH_2 (7,8)], 3.89 [2 H, d, *J* 7.8, CH_2 (4,6)], 4.96–5.10 [2 H, d, *J* 7.8, CH_2 (4,6)], 5.30 [1 H, d *J* 7.8, CH_2 (2)], 6.30 [1 H, d, *J* 7.8, CH_2 (2)]; δ_C ($CDCl_3$) 27.97 (CH_3), 28.9 [CH_2 (7)], 30.4 [CH_2 (8)], 51.1 [CH_2 (4,6)], 60.0 [CH_2 (2)], 82.2 [$C(CH_3)_3$], 85.1 [C(5)], 170.0 (C=O); *m*/*z* (CI) 349 ([M$^+$], 1.5%), 276 ([M − $[OC(CH_3)_3]$]$^+$, 44).

5-(2-Carboxyethyl)-1,3,5-trinitrohexahydropyrimidine 14

Compound **13** (1.82 g, 5.21 mmol%) was dissolved in TFA (2 ml) and after 5 min the crude product precipitated. TFA was removed by co-evaporation with DCM. The crude solid **14** was dissolved in a minimum of methanol and the solution was concentrated to an oil, which on storage crystallised to give colourless, transparent plates of the title compound **14** (1.38 g, 90%), mp 154 °C (Found: C, 28.73; H, 3.78; N, 23.77. $C_7H_{11}N_5O_8$ requires C, 28.88; H, 3.12; N, 24.05%); λ_{max}/nm 237.6 (ε 9254 dm^3 mol^{-1} cm^{-1}); ν_{max} (KBr disc)/cm^{-1} 3446.0 (C–OH), 1710.7 (C=O), 1560.5 (NNO_2 sym), 1534.8 (NO_2 asym), 1458.4, 1414.0 (NO_2 sym), 1394.7, 1344.4 (NNO_2 sym); δ_H (CD_3OD) 2.15–2.40 [4 H, m, CH_2 (7,8)], 3.89 [2 H, d *J* 7.8, CH_2 (4,6)], 4.95 [1 H, d, *J* 7.8, CH_2 (2)], 5.21 [2 H, d, *J* 7.8, CH_2 (4,6)], 6.66 [1 H, d, *J* 7.8, CH_2 (2)]; δ_C [$(CD_3)_2SO$] 27.8 [CH_2 (7)], 29.7 [CH_2 (8)], 51.2 [CH_2 (4,6)], 60.1 [CH_2 (2)], 86.0 (CNO_2), 173.2 (CO_2H); *m*/*z* (CI) 292 ([M − 1]$^+$, 28%), 276 ([M − OH]$^+$, 38), 247 ([M − CO_2]$^+$, 20).

X-Ray crystal data for 14. $C_7H_{11}N_5O_8$; M = 293.21, crystallises from methanol as clear blocks; crystal dimensions 0.50 × 0.45 × 0.18 mm. Orthorhombic, *a* = 12.331(2), *b* = 13.205(5), *c* = 14.693(4) Å, *V* = 2392.5(12) Å^3, *Z* = 8, D_c = 1.628 Mg m^{-3}, space group *Pbca* (D_2^{15}, No. 61), Mo-Kα radiation (λ = 0.710 69 Å), μ(Mo-Kα) = 0.149 mm^{-1}, *F*(000) = 1216.

Three-dimensional, room temperature X-ray data were collected in the range 3.5° < 2θ < 45° on a Siemens P4 diffractometer by the omega-scan method. Of the 2027 reflections measured, all of which were corrected for Lorentz and polarisation effects, but not for absorption, 1189 independent reflections exceeded the significance level $|F|/\sigma(|F|) > 4.0$. The structure was solved by direct methods and refined by full-matrix least-squares methods. Hydrogen atoms were detected and refined in riding mode. Refinement converged at a final *R* = 0.0619 (w*R*2 = 0.2401, 181 parameter, mean maximum δ/σ 0.000, 0.000), with allowance for the thermal anisotropy of all non-hydrogen atoms. Minimum and maximum final electron density −0.525 and 0.609 e Å^{-3}. A weighting scheme $w = 1/[\sigma^2(F_o^2) + (0.0665*P)^2 + 4.3310*P]$ where $P = (F_o^2 + 2*F_c^2)/3$ was used in the latter stages of refinement. Complex scattering factors were taken from the program package[21] SHELXL97 as implemented on the Viglen 486dx computer.

† CCDC reference number 207/380. See http://www.rsc.org/suppdata/p1/a9/a906884h/ for crystallographic files in .cif format.

5-(3′-Hydroxypropyl)-1,3,5-trinitrohexahydropyrimidine 15

Compound **13** (0.65 g, 1.88 mmol) in THF (40 ml) was added to a suspension of $NaBH_4$ (0.09 g, 2.41 mmol) and LiI (0.33 g, 2.46 mmol) in THF (15 ml) at 0 °C. The reaction mixture was stirred for 2 h at rt and then quenched by addition of ethanol followed by water, and the THF layer was separated. The aqueous layer was back-extracted with DCM and the combined organic layers were dried over $MgSO_4$, filtered, and evaporated to give a crude white solid. Purification by flash column chromatography (EtOAc, R_f 0.25) afforded the title compound **15** as a white powder (0.21 g, 40%) (Found: C, 30.91; H, 4.83; N, 25.34. $C_7H_{13}N_5O_7$ requires C, 30.11; H, 4.69; N, 25.08%); λ_{max}/nm 238.4 (ε 3553.8 dm^3 mol^{-1} cm^{-1}); ν_{max} (KBr disc)/cm^{-1} 3444.6 (OH), 1559.3 (NNO_2 asym, str), 1453.1 (NO_2 sym), 1282.7 (NNO_2 sym), 1243.2 (NO_2 sym); δ_H [$(CD_3)_2SO$] 1.25–1.50 [2 H, m, CH_2 (1′)], 1.90–2.15 [2 H, m, CH_2 (2′)], 3.25–3.48 [2 H, m, CH_2 (3′)], 4.09 [2 H, d, *J* 7.8, CH_2 (4,6)], 4.60 (1 H, t, *J* 4.0, OH), 5.13 [1 H, d, *J* 7.8, CH_2 (2)], 5.30 [2 H, d, *J* 7.8, CH_2 (4,6)], 6.84 [1 H, d, *J* 7.8, CH_2 (4,6)]; δ_C ($CDCl_3$) 25.4 [CH_2 (1′)], 31.6 [CH_2 (2′)], 51.0 [CH_2 (4,6)], 59.8 [CH_2 (3′)], 60.2 [CH_2 (2)], 86.0 [C (5)]; *m*/*z* (EI) $[M - 1]^+$ 278 (11%), [$M - HNO_2$] 231 (13).

5-(2′-Cyanoethyl)-1,3,5-trinitrohexahydropyrimidine 16

Amberlyst A-21 (2.20 g) and **10** (0.5 g, 2.3 mmol) were stirred together for 5 days in acrylonitrile (15 ml), resin was removed by filtration, and the solvent was evaporated off. The product was dissolved in acetone and the solution concentrated to afford an orange oil containing some white solid. Purification was carried out by repeated crystallisation from acetone to afford the *title compound* **16** as a white solid (0.4 g, 64%), mp 146 °C (Found: C, 30.69; H, 3.38: N, 29.75. $C_7H_{10}N_6O_6$ requires C, 30.65: H, 3.68: N, 30.66%); λ_{max}/nm 214.4, 238.9 (ε 1772, 1639 dm^3 mol^{-1} cm^{-1}); ν_{max}/cm^{-1} 1578 (NNO_2 asym), 1558 (NO_2 asym), 1457, 1439 (NO_2 sym); δ_H [$(CD_3)_2SO$] 2.33 (2 H, t, *J* 15.0, 7.8, C$H_2$$CH_2$CN), 2.75 (2 H, t, *J* 15.0, 7.8, CH_2CH_2CN), 4.05 [2 H, d, *J* 15.0, CH_2 (4,6)], 5.05 [1 H, d *J* 15.0, CH_2 (2)], 5.35 [2 H, d, *J* 15.0, CH_2 (4,6)], 6.97 [1 H, d *J* 15.0, CH_2 (2)]; δ_C ($CDCl_3$) 10.8 [CH_2 (8)], 29.0 [CH_2 (7)], 50.3 [CH_2 (4,6)], 59.4 [CH_2 (2)], 84.4 [C (5)], 119.2 (CN); *m*/*z* (CI) 275 ([M^+], 25%).

5-(2′-Cyanoethyl)-1,5-dinitro-1,4,5,6-tetrahydropyrimidine 17

Conc. H_2SO_4 (0.24 g) was added dropwise to a stirred suspension of lithium aluminium hydride (0.19 g, 5.6 mmol) in THF (3 ml) under argon at 0 °C.[17] The resulting mixture was stirred 1 h at 0 °C and then compound **16** (0.43 g, 1.6 mmol) in THF (20 ml) was added dropwise. The reaction mixture was brought to rt, stirred overnight, and quenched at 0 °C by dropwise addition of ice–water until the suspension clarified. Aq. sodium hydroxide (1.0 g in 7 ml) was added to precipitate hydrated aluminium oxide, and the mixture was extracted with DCM. The organic layer was dried over $MgSO_4$, filtered, and concentrated to afford a crude white solid (0.28 g). The *title compound* **17** was isolated by flash column chromatography (R_f 0.3, ether) as a dewy oil (50 mg, 14%) (Found: C, 37.01; H, 3.99; N, 30.83. $C_7H_9N_5O_4$ requires C, 37.44; H, 4.08; N, 29.02%); δ_H ($CDCl_3$) 2.40–2.60 [4 H, m, CH_2 (7,8)], 4.50 [2 H, s, CH_2 (4)], 5.89 [2 H, s, CH_2 (6)], 7.25 (1 H, s, CH); δ_C ($CDCl_3$) 16.2 [CH_2 (7)], 27.8 [CH_2 (8)], 48.7 [CH_2 (4)], 59.5 [CH_2 (6)], 121.3 [CH_2 (2)]; *m*/*z* (EI) 227 ([M^+], 16%).

General procedure for conjugation of a hapten to a carrier protein

Compound **12** as a typical hapten, KLH as a typical carrier protein. Compound **12**, (16.5 mg, 45 mmol) was dissolved in water/DMF (1 : 1, v/v) and a solution of EDC (13.0 mg, 68 mmol) in water (100 µl) was added, followed by sulfo-NHS (15.0 mg, 68 mmol) in water (100 µl). The resulting solution was stirred for 30 min and then added to a solution of KLH (20 mg) in water (2 ml). Stirring was continued overnight at rt. The derivatised carrier was dialsed for 3 h against 3 changes of HEPES buffer (50 mM; pH 8.0; 0.15 M NaCl) and then against phosphate-buffered saline (pH 6.8) overnight, and stored at 4 °C prior to murine immunisations.

Acknowledgements

This work was supported by a grant from DERA and is published under © British Crown Copyright.

References

1 G. R. J. Thatcher and H. Weldon, *Chem. Soc. Rev.*, 1998, **27**, 331.
2 R. M. J. Palmer, A. G. Ferrige and S. Moncada, *Nature* (*London*), 1987, **327**, 524; L. J. Ignarro, G. M. Buga, K. S. Wood, R. E. Byrns and G. Chaudhuri, *Proc. Natl. Acad. Sci. USA*, 1987, **84**, 9265, A. R. Butler and D. L. H. Williams, *Chem. Soc. Rev.*, 1993, **22**, 233; B. Mayer, S. Pfeiffer and B. Hemmens, *Angew. Chem.*, *Int. Ed.*, 1999, **38**, 1714.
3 F. J. Di Carlo and L. C. Holmes, *J. Chem. Educ.*, 1972, **49**, 726.
4 G. F. Henning, *Ger. Pat.* 104 280, 1899.
5 Poudreries de Belgique, Soc. An., *Belg. Pat.* 488 943, 1949 (*Chem. Abstr.*, 1949, **48**, 4760*b*); T. Urbanski, *Chemistry and Technology of Explosives*, Pergamon Press/PWN - Polish Scientific Publishers, Warsaw, 1st edn., 1967, vol. 1, p. 87.
6 E. von Herz, *Brit. Pat.* 145 793, 1921; *U.S. Pat.* 1 402 693, 1922 (*Chem. Abstr.*, 1922, **16**, 1014).
7 G. C. Hale, *J. Am. Chem. Soc.*, 1925, **47**, 2754.
8 (*a*) D. R. Burton and C. F. Barbas, III, *Advances in Immunology*, ed. F. J. Dixon, Academic Press, San Diego, 1994, vol. 57, p. 191; (*b*) M. Sela, *Science*, 1969, **166**, 1365.
9 S. Sell, *Immunology*, *Imunopathology and Immunity*, Elsevier, New York, 1987, p. 69.
10 A. T. Camp, N. S. Marans, D. E. Eldrick and R. F. Preckel, *J. Am. Chem. Soc.*, 1955, **77**, 751.
11 N. S. Marans, D. E. Eldrick and R. F. Preckel, *J. Am. Chem. Soc.*, 1954, **76**, 1304.
12 C. S. Choi and H. Prince, *Acta Crystallogr.*, *Sect. B*, 1972, **28**, 2857.
13 H. Ritter and H. H. Licht, *Prop. Explos. Pyrotech.*, 1985, **10**, 147.
14 R. Ballini and S. Rosini, *Synthesis*, 1987, 711.
15 J. Kollonitsch, O. Fuchs and V. Gabor, *Nature* (*London*), 1954, **173**, 125; H. C. Brown, S. Narasimhan and Y.-M. Choi, *J. Org. Chem.*, 1982, **47**, 4702.
16 K. Landsteiner, in *The Specificity of Serological Reactions*, Harvard University Press, Cambridge, MA, 1945.
17 H. C. Brown and N.-M. Yoon, *J. Am. Chem. Soc.*, 1968, **90**, 2927.
18 C. Holstead and A. H. Lamberton, *J. Chem. Soc.*, 1952, 1886.
19 J. V. Staros, R. W. Wright and D. M. Swingle, *Anal. Biochem.*, 1986, **156**, 220.
20 S. Nicklin and A. Hutchinson, unpublished results.
21 G. M. Sheldrick, SHELXL97, An Integrated System for Solving and Refining Crystal Structures from Diffraction Data, University of Göttingen, Germany, 1997.

Paper a906884h