Pergamon

Tetrahedron Vol. 51, No. 43, pp. 11815-11830, 1995

0040-4020/95 $9.50+0.00

0040-4020(95)00743-1

(17-Tetrabenzo[*a,c,g,i*]fluorenyl)methylchloroformate (TbfmocCl) a Reagent for the Rapid and Efficient Purification of Synthetic Peptides and Proteins

Angus R. Brown, Stephen L. Irving, Robert Ramage* and Gilles Raphy

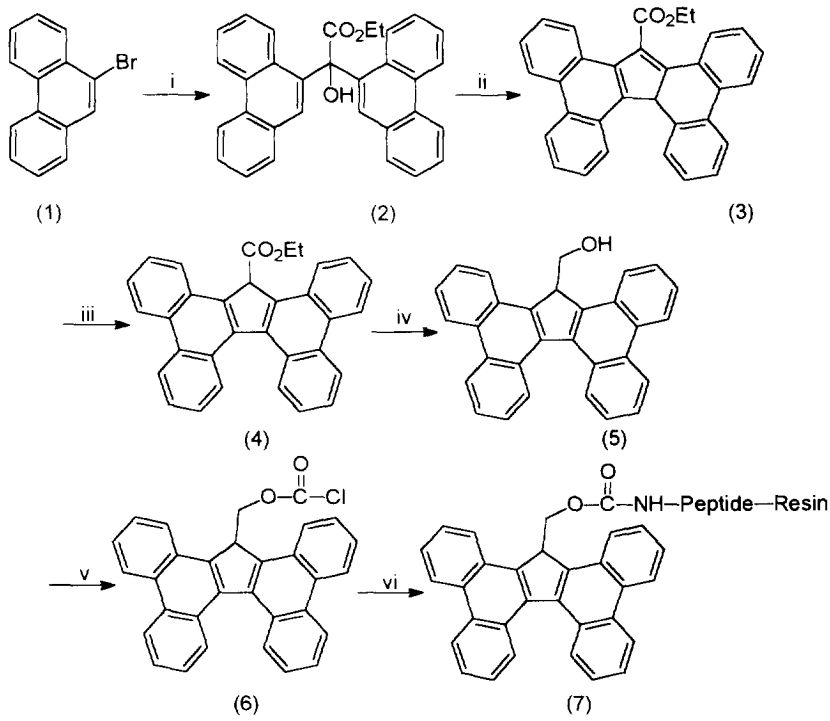
Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh, Scotland, EH9 3JJ.

Abstract: The optimised synthesis of 17-tetrabenzo[*a,c,g,i*]fluorenylmethylchloroformate (TbfmocCl) and its use a reagent for the rapid and efficient purification of synthetic polypeptides is reported. Incorporation of the Tbfmoc group onto the N-terminus of resin bound peptides allows purification of the cleaved labelled peptide either by affinity binding to porous graphitised carbon or as a hydrophobic chromatographic probe to simplify HPLC purification of peptides and small proteins.

Although the synthesis of peptides on a solid support has developed into a highly sophisticated science, largely due to the increasing demand for biologically active peptide targets, the product obtained on release from the resin is seldom pure. This may be due to either side reactions which occur during assembly and cleavage[1], or due to incomplete coupling steps. For short sequences of less than 30 amino acids often the crude cleaved material may contain only a minor amount of contaminants but nevertheless, one or more purification steps are required to obtain the peptide in pure form. In the case of large polypeptides, the main obstacle to the success of such syntheses has always been the difficulty in separating the desired sequence from truncated products which can accumulate on the resin during synthesis. The physical and chemical similarities of the desired sequence and the shorter truncated impurities can often require protracted purification in order to isolate the target sequence in pure form[2].

Following early work by Merrifield[3] several methods have been reported which allow differentiation between the target sequence and any acetylated truncations by derivatising the N-terminus of the resin bound polypeptide with a group, which then allows separation of the desired sequence from the acetylated truncations either by affinity type binding[4,5] or covalent attachment[6] to a solid support. It must be emphasised that a prerequisite of all techniques involving N-terminal derivatisation is the inclusion of a capping step after each coupling cycle in order to block the amino termini of any deletion sequences. Several groups have reported the use of reversible hydrophobic probes to simplify the chromatographic purification of peptides[7] and proteins[8].

We recently gave a brief report of the use of the base labile N^{α}-protecting group, tetrabenzo[*a,c,g,i*]fluorenyl-17-methoxycarbonyl (Tbfmoc) (7), for both affinity purification of polypeptides on porous graphitised carbon (PGC)[9,10] and also as hydrophobic chromatographic probe to simplify the purification of peptides by RP-HPLC[10].



Scheme 1. i) Mg, THF; $(CO_2Et)_2$. ii) TFA/CH_2Cl_2. iii) Et_3N/CH_2Cl_2. iv) Diisobutylaluminium hydride/CH_2Cl_2. v) Triphosgene/N,N-dimethylaniline/CH_2Cl_2. vi) Peptide-Resin/diisopropylethylamine/CH_2Cl_2 or 1,4-dioxane.

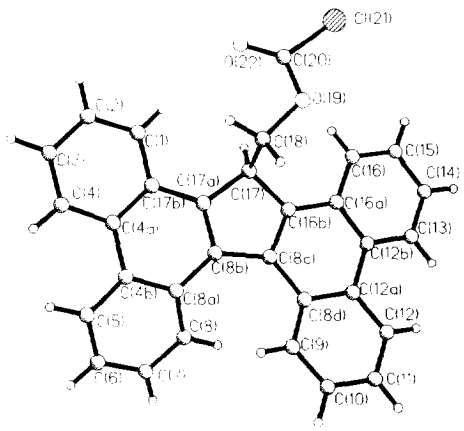
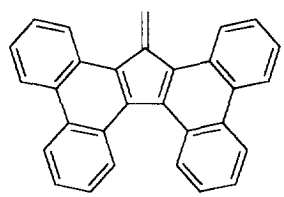


Figure 1. Molecular structure of **6**[11].

The synthesis of the key reagent, chloroformate (6) Scheme 1, has been optimised to allow the synthesis of multi-gram quantites without the need for chromatographic purification of any of the intermediates (2) to (6). Alcohol (2) was obtained from the rection of 9-phenanthrylmagnesium bromide with diethyl oxalate in THF, and on treatment with TFA in dichloromethane (2) underwent a [2+2] Nazarov-type cationic cyclisation to give mainly the 8*b*-H isomer (3)[12]. Isomerisation of (3) to (4) occurred rapidly on treatment with triethylamine and, after reduction of (4), 17-tetrabenzo[*a,c,g,i*]fluorenylmethanol (5) was obtained in an overall yield of 41%. The crystalline chloroformate (6)[11] (Figure 1) was then obtained in good yield by treating (5) with an excess of triphosgene[13] in the presence of N,N-dimethylaniline. The Tbfmoc group is then introduced onto the N-terminus of the resin bound peptide sequence by manually treating with an excess of chloroformate (6) in the presence of DIEA in a sonic bath. After coupling of the Tbfmoc group, the labelled peptide is then cleaved from the resin by treatment with TFA/scavengers and the peptide can then be purified by one of two methods.

Method A- Affinity purification on porous graphitised carbon (PGC). PGC has been developed as an inert and robust chromatographic support, although for our purposes it is also useful for aiding the purification of Tbfmoc-labelled peptides due to the fact that it adsorbs large flat aromatic groups with high affinity[14]. Hence the labelled peptide can be bound to the PGC and the impurities are then simply washed away. The base lability of the Tbfmoc N^{α}-protecting group then allows facile release of the pure desired polypeptide sequence by treatment with base. Recent studies[10,12] have shown that for short relatively hydrophilic sequences, the desired sequence can be eluted from the PGC by treatment with 10% piperidine in aqueous acetonitrile (70%). However, for longer or more hydrophobic sequences we recommend that the polypeptide is eluted from the PGC by treating with a mixture of 10% piperidine in 1:1 6M guanidine hydrochloride/isopropanol. The graphite can then be regenerated by washing with hot 1,4-dioxan to remove the 17-methylenetetrabenzo[*a,c,g,i*]fluorene (**8**) and the corresponding piperidine adduct.



(**8**)

Method B- Reverse phase-HPLC based chromatographic purification. The hydrophobicity of the Tbfmoc group results in the labelled peptide sequence eluting later from reverse phase HPLC columns than the non-labelled deletion sequences. The specific UV/Vis absorbance of the Tbf ring system (λ_{max}364 nm) also allows the unequivocal identification of the desired labelled sequence by monitoring the eluent from the HPLC at this wavelength. Hence the Tbfmoc-peptide sequence can be identified and isolated before the Tbfmoc N^{α}-protecting group is removed by treatment with base and desalted to give essentially pure peptide. In order to

demonstrate the viability of both these methods, A and B, we have constructed several biologically important polypeptide sequences of varying length and complexity, and have purified each sequence to homogeneity.

α-Calcitonin Gene Related Peptide (8-37). This 30 residue peptide amide was synthesised on a 0.25 mmol scale using our previously reported amide linker[15]and single coupling each residue as its HOBt ester. In order to test the purification protocol **no attempt was made to optimise any of the couplings** and the progress of the assembly, as assesed by UV monitoring of the eluent from each Fmoc deprotection step[16], is illustrated in figure **2**.

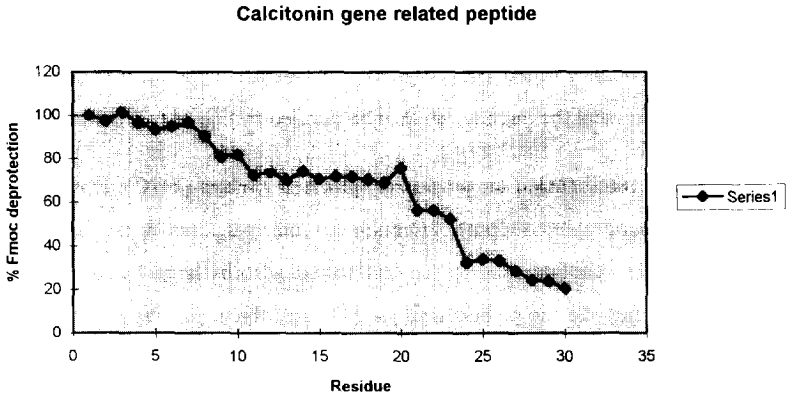


Figure 2. Progress of the assembly of α-CGRP (8-37)

At the completion of the assembly phase the Fmoc deprotection for valine-8 was 20% of that obtained for the starting resin, and indicated the presence of several deletion products on the resin. The total resin bound product was therefore treated with TbfmocCl (**6**) before being cleaved and purified by affinity binding of the Tbfmoc-labelled peptide to PGC (method A). Figure **3** shows the RP-HPLC trace of the crude cleaved Tbfmoc-labelled peptide before PGC purification, and confirmed the presence of several truncated deletion products. However, the presence of one major Tbfmoc-labelled species was also confirmed by re-running the HPLC when monitoring at the chromophoric wavelength of the tetrabenzofluorene ring system (364 nm) (Figure **3**). The crude cleaved mixture was then mixed with PGC to allow the labelled peptide to affinity bind, and then the unlabelled impurities were removed by repeated washing with 6M guanidine hydrochloride/isopropanol (1:1). Finally the desired α-CGRP (8-37) (Figure **3**) was eluted from the PGC by treating with 10% piperidine in 6M guanidine hydrochloride/isopropanol (1:1).

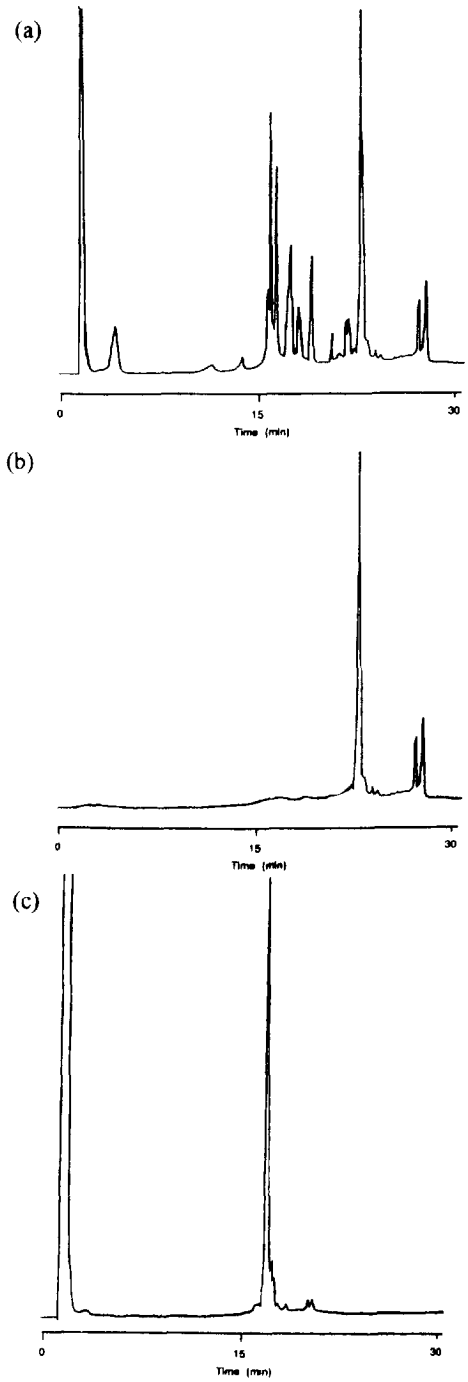


Figure 3. HPLC of α-CGRP (8-37) (a) crude cleaved peptide at 214 nm. (b) crude cleaved peptide at 364 nm. (c) crude peptide after PGC purification (method A)

MBD from MeCP2[17]. We have also successfully utilised both methods A and B to purify the 85 amino acid methylated DNA binding domain (MBD) from the chromosomal protein MeCP2. Figure 4 shows the crude Tbfmoc-labelled MBD after cleavage and also after HPLC based purification to give the pure fully biologically active protein[17].

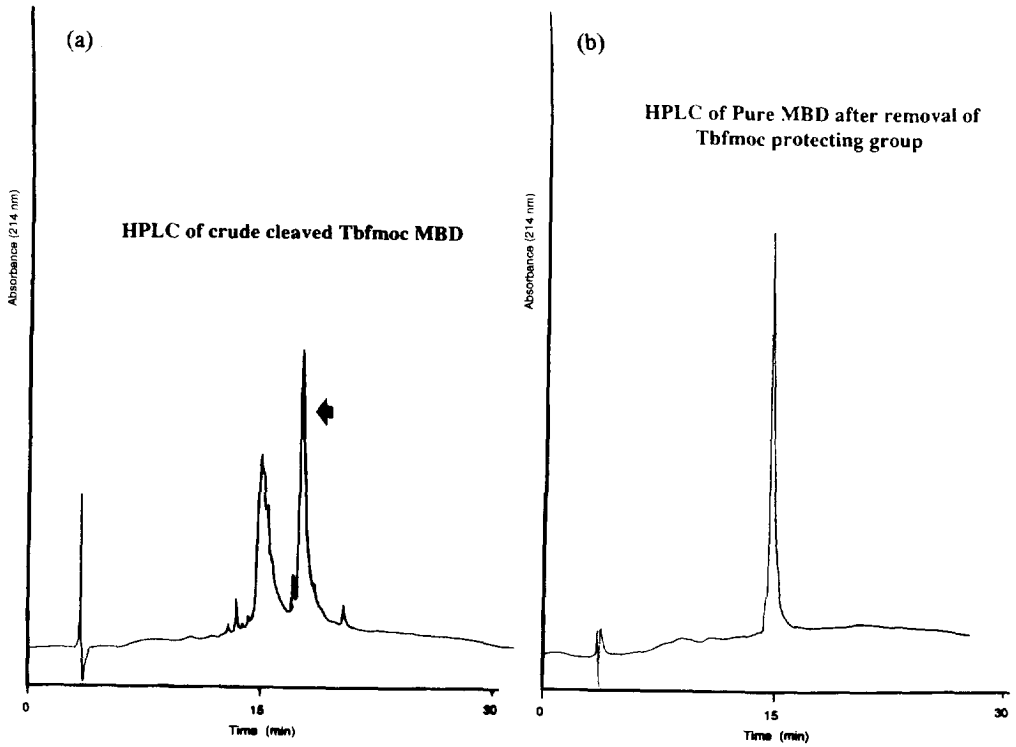


Figure 4. HPLC of (a) crude cleaved Tbfmoc-MBD (Tbfmoc labelled peak arrowed) and (b) Pure MBD.

Restriction Alleviation Protein[18]. The use of the Tbfmoc group as a hydrophobic chromatographic probe, has also allowed us to investigate milder conditions for removal of the Tbfmoc N^{α}-protecting group in solution. Synthetic studies on the predicted ral gene product from bacteriophage λ had previously demonstrated the effectiveness of our Tbfmoc methodology in allowing the rapid purification of Acm protected cysteine containing proteins[10]. However, the removal of the Acm thiol protection did not proceed smoothly in our hands. We therefore re-synthesised the 66 amino acid Ral protein incorporating the 4 cysteine residues present as the acid labile trityl derivative. At the completion of the assembly the Tbfmoc group was introduced onto the N-terminus of the resin bound product and then a portion of this was cleaved to give the Tbfmoc-labelled Ral protein (Figure 5). The labelled protein was then isolated by HPLC, monitoring at 364 nm, and subsequent lyophilisation yielded the pure Tbfmoc-protein. Treatment of the Tbfmoc-labelled Ral with 6M guanidine

hydrochloride (pH 8.5, 0.1M Tris) containing dithiothreitol at 37°C resulted in the smooth removal of the Tbfmoc group within 4 hours (Figure **6**), to yield the fully reduced protein after desalting. The general utility of this method has also been demonstrated by the recent application of this methodology to the synthesis of the biologically significant β-chemokine MCP-1[19].

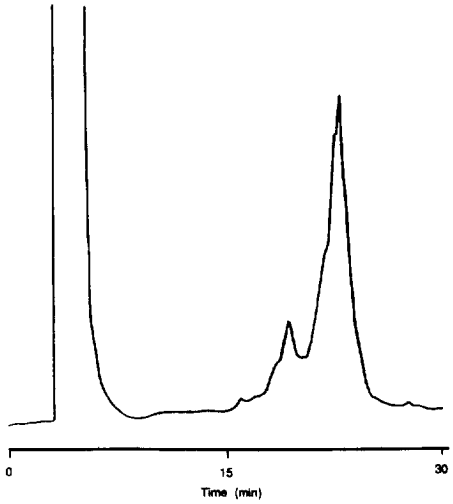


Figure 5. HPLC of crude cleaved Tbfmoc labelled Ral protein at 214 nm.

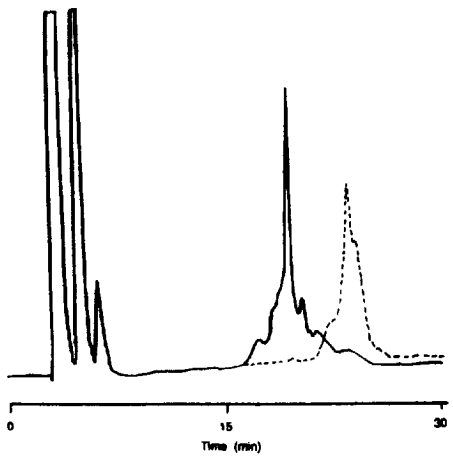


Figure 6. HPLC of Tbfmoc-Ral deprotection (6M Gdm.HCl, pH 8.5). Broken line = 364 nm, t = 0; Solid line = 214 nm t = 4 hrs.

In conclusion we have demonstrated that the crystalline reagent 17-tetrabenzo[*a,c,g,i*]fluorenylmethyl chloroformate (**6**) is easily synthesised and, when used to introduce the Tbfmoc group onto the N-terminus of resin bound peptides and small proteins, can simplify the purification of these compounds. We also find that our Tbfmoc/PGC methodology is particularly useful for the purification of hydrophobic and/or difficult sequences which often contain truncated deletion sequences.

EXPERIMENTAL

General. All amino acids, tricyclic amide linker functionalised resin[15] and the 4-alkoxybenzylalcohol funtionalised poystyrene resin were purchased from Bachem, Bubendorf, Switzerland. The following side chain protecting groups were used: Arg(Pmc); Asp(OBu^t); Asn(Trt); Cys(Trt); Gln(Trt); Glu(OBu^t); His(Trt); Lys(Boc); Ser(Bu^t); Thr(Bu^t) and Tyr(Bu^t). Peptide synthesis grade DMF, 1,4-dioxan and piperidine were purchased from Rathburn Chemicals, Walkerburn, Scotland. Peptide synthesis grade TFA was purchased from Applied Biosystems Ltd. All remaining chemicals were obtained from Aldrich. Melting points were determined in open capillaries and are uncorrected. Thin layer chromatography (TLC) was performed with silica gel 60GF-254 (Merk 5735) on plastic or aluminium sheets. Ultra violet spectra were recorded on a Cary 210 spectrophotometer.Nuclear magnetic resonance spectra were recorded on either a Brüker WP 80 (80 MHz), WP 200 (200 Mhz) or WH 360 (360 Mhz) machine in the solvent indicated, and chemical shifts were measured relative to tetramethylsilane as the external standard. Fast atom bombardment (FAB) mass spectra were obtained using a Kratos MS50TC instrument and laser desorption time of flight mass spectra were obtained using a PerSeptive Biosystems LaserTec Benchtop II system. Amino acid analysis was carried out on an LKB 4151 Alpha Plus amino acid analyser equipped with an LKB 2220 Recording Integrator. Gel filtration was carried out using a Microperpex 2132 peristaltic pump and two LKB Uvicord 2138s Spectrophotometers at 277 and 365 nm. HPLC was performed using an Applied Biosystems 151A HPLC system.

Solid Phase Peptide Synthesis. All polypeptides were synthesised using the 9-fluorenylmethoxycarbonyl (Fmoc) strategy of N$^\alpha$ protection on an Applied Biosystems 430A peptide synthesiser fitted with a UV monitoring system as described previously[16]. All amino acids were double-coupled, unless otherwise stated, as the symmetrical anhydride (1mmol of amino acid) followed by the HOBt active ester (0.5 mmol of amino acid). The exceptions to this were the amino acids asparagine, glutamine and histidine, which were coupled twice *via* their HOBt esters and glycine, which was coupled singly as a symmetrical anhydride (2 mmol of amino acid). Synthetic proceedures were pre-programmed into the ABI 430A synthesiser prior to the comencement of synthesis using our own optimised in-house synthetic cycles. Each synthetic cycle involved

(1) a capping step, (2) deprotection of the base labile Fmoc group, (3) coupling of the next protected amino acid. The pre-programmed synthetic cycles are summarised below.

1. Capping - The resin was vortexed with a solution of acetic anhydride (0.5M), DIEA (0.125M) and HOBt (0.2% w/v) in DMF (10 ml) for 10 minutes, before the capping solution was drained from the vessel and the resin washed by six portions of DMF.

2. Deprotection - The resin was vortexed with a solution of 20% piperidine/DMF for 3 minutes before being drained. An aliquot of the deprotection solution was then sent to a UV detector in order to quantify the amount of fulvene-piperidine adduct present and hence subsequently gave an indication of the percentage incorporation of each residue. Deprotection was then repeated for a second time for 1 minute in order to establish if the N^{α} protecting group had been completely removed. Finally the resin was washed with six portions of DMF/1,4-dioxan (1;1).

3. Coupling - Double couple cycles. The resin was vortexed with a solution of 0.5mmol Fmoc amino acid preformed symmetrical anhydride (formed from 1mmol Fmoc AA and 0.5mmol DIC in the activator vessel). The first coupling cycle was allowed to continue for 30 minutes before the solution was drained from the reaction vessel and the resin washed with two portions of DMF. The resin was then vortexed for a second 30 minute period with 0.5mmol of Fmoc amino acid HOBt active ester (preformed from 0.5 mmol Fmoc AA, 0.5 mmol HOBt and 0.5 mmol DIC) before being drained and washed with four portions of DMF. Single couple cycles. As in the second coupling of the double couple cycle above, but with the quantities of Fmoc AA, HOBt and DIC increased to give 1mmol of HOBt active ester.

Ethyl (*bis*-phenanthren-9-yl)glycolate (2).

9-Bromophenanthrene (100 g, 0.389 mol) was dissolved in dry THF (200 ml) and slowly added to Mg turnings (10 g, 0.41 mol), together with a crystal of iodine under an atmosphere of dry nitrogen. After initiation of the reaction, the mixture was cooled on an ice-bath to control the initial vigour of the reaction, then stirred at room temperature for 45 minutes. The mixture was cooled on an ice/salt bath and diethyl oxalate (23.7 g, 0.187 mol) added dropwise over a period of 10 minutes. The mixture was then stirred at room temperature for 1.5 h, after which time ice, then aqueous 3.7M NH_4Cl (250 ml) was added. The mixture was stirred for 10 minutes and extracted with dichloromethane (2 x 500 ml). A precipitate formed in the organic extract and this was filtered off and disguarded. The filtrate was dried ($MgSO_4$) and the solvent removed *in vacuo* to leave a yellow oil which was triturated with ether and allowed to stand overnight. The precipitated solid was filtered off and recrystallised from DCM/n-hexane to give the *title compound* as a white solid (48.8 g, 57%);

m.p. 189-192^{0}C; Found: C, 84.4; H, 5.36. $C_{32}H_{24}O_3$ requires C, 84.2; H, 5.26%; TLC: R_f 0.5 ($CHCl_3$): δ_H(360 Mhz, $CDCl_3$): 8.79 (2H, d, J=8.3 Hz, aromatic), 8.72 (2H, d, J=8.5 Hz, aromatic), 8.53 (2H, d, J=8.0 Hz, aromatic), 7.70-7.47 (12H, m, aromatic), 4.51 (1H, s, OH), 4.42 (2H, q, J=7.2 Hz, CH_2), 1.18 (3H, t, J=7.2 Hz, CH_3); δ_C (50 MHz, $CDCl_3$): 175.7 (CO,ester), 135.0, 131.4, 130.6, 130.5, 130.1 (quaternary aromatic C's),

129.2, 128.2, 127.3, 126.6, 126.2, 126.1, 122.9, 122.3 (aromatic CH's), 84.3 (C_1, quaternary), 62.9 (CH_2), 13.8 (CH_3); ν_{max} (DCM): 3500 (OH), 3060 (aromatic CH), 1730 (ester C=O), 1600 cm^{-1} (aromatic C=C); λ_{max}(DCM) 300 (ε 20200 $dm^3mol^{-1}cm^{-1}$), 289 (18900), 278 (25900), 257 nm (103000); m/z (EI): 456 (M^+), 364, 363; HRMS: 456.1739, $C_{32}H_{24}O_3$ requires 456.17253.

17-(Ethoxycarbonyl)-8*b*H-tetrabenzo[*a,c,g,i*]fluorene (3).

Ethyl (*bis*-phenanthren-9-yl)glycolate (27 g, 59.2 mmol) was taken in dichloromethane (270 ml) and TFA (81 ml) was slowly added with stirring. After 20 minutes, the solvent was removed *in vacuo* and the residue triturated with ether to give the *title compound* as a yellow solid (23.1 g, 89%);

m.p. 161-163^{0}C Found: C, 86.5; H, 5.12. $C_{32}H_{22}O_2$ requires C, 87.7; H, 5.02%^{20}; TLC: R_f 0.69 ($CHCl_3$): δ_H(360 Mhz, $CDCl_3$): 8.81-8.74 (2H, m, aromatic), 8.12-7.92 (3H, m, aromatic), 7.82-7.61 (6H, m, aromatic), 7.47-7.29 (4H, m, aromatic), 7.11-7.06 (1H, m, aromatic), 5.38 (1H, s, CH), 4.62-4.49 (2H, m, CH_2), 1.41 (3H, t, J=7.2Hz, CH_3); δ_C(50 Mhz, $CDCl_3$): 168.5 (ester C=O), 146.9 (quarternary aromatic C), 138.3-123.2 (aromatic C's), 61.7 (CH_2), 52.5 (CH), 13.9 (CH_3); ν_{max} (DCM):2960-2900 (CH), 1739 (ester C=O), 1610 cm^{-1} (aromatic C=C); λ_{max}(DCM) 372 (ε 10300 $dm^3mol^{-1}cm^{-1}$),301 (32200), 290 (31200), 254 nm (66500), m/z (EI): 438 (M^+), 364, 363; HRMS: 438.1610, $C_{32}H_{22}O_2$ requires 438.16197.

17-(Ethoxycarbonyl)tetrabenzo[*a,c,g,i*]fluorene (4).

17-(Ethoxycarbonyl)-8*b*H-tetrabenzo[*a,c,g,i*]fluorene (15 g, 43.2 mmol) was taken in dichloromethane (100 ml) and triethylamine (0.2 ml, 1.44 mmol) added with swirling. The solvent was then removed *in vacuo* and the residue triturated with ether to give the *title compound* as a yellow solid (14.5 g, 97%);

m.p. 168-169^{0}C Found: C, 87.1; H, 5.02. $C_{32}H_{22}O_2$ requires C, 87.7; H, 5.02%^{20}; TLC: R_f 0.67 ($CHCl_3$): δ_H(360 Mhz, $CDCl_3$): 8.76-8.63 (6H, m, aromatic), 8.27-8.24 (2H, m, aromatic), 7.70-7.57 (8H, m, aromatic), 5.50 (1H, s, CH), 4.04 (2H, q, J=7.2 Hz, CH_2), 0.96 (3H, t, J=7.2 Hz, CH_3); δ_C(50 Mhz, $CDCl_3$): 172.6 (ester C=O), 138.8, 138.7, 132.2, 131.0, 129.2, 128.3 (quarternary aromatic C), 128.2, 127.8, 126.8, 126.7, 125.6, 124.5, 123.8, (aromatic CH's), 62.1 (CH_2), 55.4 (CH), 14.5 (CH_3); ν_{max} (DCM):3080 (aromatic CH), 2930 (aliphatic CH), 1725 (ester C=O), 1610 cm^{-1} (aromatic C=C); λ_{max}(DCM) 386 (ε 15200 $dm^3mol^{-1}cm^{-1}$), 370 (17500), 302 (47500), 290 (37800), 279 (32300), 262 (71400), 253 nm (76500), m/z (EI): 438 (M^+), 364, 363; HRMS: 438.1633, $C_{32}H_{22}O_2$ requires 438.16197.

17-Tetrabenzo[*a,c,g,i*]fluorenylmethanol (5).

17-(Ethoxycarbonyl)tetrabenzo[*a,c,g,i*]fluorene (6.0 g, 13.7 mmol) was taken in dichloromethane (300 ml) in an atmosphere of dry nitrogen and cooled to -65 °C. A solution of 1M diisobutylaluminium hydride (41.4 ml, 41.4 mmol) in dichloromethane was added dropwise, such that the temperature remained below -60°C. The mixture was then stirred at -70°C for 1 h and for a further 2 h at room temperature. The mixture was cooled to -30°C and

20% aqueous acetic acid (90 ml) carefully added. The mixture was diluted with water (100 ml) and extracted with dichloromethane (300 ml + 2 x 200 ml), the organic extract dried ($MgSO_4$) and solvent removed *in vacuo* to give a yellow solid which was washed with ether to give the *title compound* (4.8 g, 88%);
m.p. 202-204°C Found: C, 90.2; H, 5.17. $C_{30}H_{20}O$ requires C, 90.9: H, 5.05%[20]; TLC: R_f 0.74 (10% MeOH in $CHCl_3$): δ_H(200 Mhz, $CDCl_3$): 8.82-8.64 (6H, m, aromatic), 8.33-8.25 (2H, m, aromatic), 7.75-7.57 (8H, m, aromatic), 5.15 (1H, t, J=4.3 Hz, CH), 4.43 (2H, d, J=4.3 Hz, CH_2); δ_C (50 Mhz, $CDCl_3$): 141.6, 1137.3, 131.4, 130.4, 128.5, 127.9 (quaternary C's), 127.4, 126.9, 126.1, 125.9, 125.0, 124.5, 123.4 (aromatic CH's), 66.5 (CH_2), 50.8 (CH); ν_{max} (DCM): 3580 (OH), 3010 (aromatic CH), 2910,2890 (aliphatic CH), 1605 cm^{-1} (aromatic C=C); λ_{max}(DCM) 380 (ε 16500 $dm^3mol^{-1}cm^{-1}$), 366 (17600), 301 (40300), 289 (33000), 261 (61500), 253 nm (66700), m/z (FAB): 396 (M^+), 379, 366; HRMS 396.15142, $C_{30}H_{20}O$ requires 396.15141.

(17-Tetrabenzo[*a,c,g,i*]fluorenyl)methyl chloroformate (6).
17-Tetrabenzo[*a,c,g,i*]fluorenylmethanol (1.0 g, 2.52 mmol) was taken in dichloromethane (20 ml) along with triphosgene (0.5 g, 1.68 mmol) and N,N-dimethylaniline (0.64 ml, 5.04 mmol) added. The mixture was stirred for 45 minutes and the precipitated solid filtered off and recrystallised from dichloromethane/n-hexane to give the *title compound* as a pale yellow solid (0.577 g). A further crop of product was obtained from the dichloromethane filtrate after adding water, acidifying to pH1 with 2M HCl, drying the organic layer ($MgSO_4$), removing the solvent and recrystallising the residue from dichloromethane/n-hexane. Total yield (0.819 g, 71%);
m.p. 194-195°C Found: C, 81.3; H, 4.22, Cl, 7.66. $C_{31}H_{19}O_2Cl$ requires C, 81.1: H, 4.14: Cl, 7.74%[20]; TLC: R_f 0.75 (DCM): δ_H(200 Mhz, $CDCl_3$): 8.80-8.75 (6H, m, aromatic), 8.63 (2H, d, J=8.0 Hz, aromatic), 8.20-7.57 (8H, m, aromatic), 5.20 (1H, t, J=5.8 Hz, CH), 4.78 (2H, d, J=5.8 Hz, CH_2); δ_C(90 Mhz, dioxane d-8): 149.5 (CO), 140.9, 136.7, 131.4, 130.3, 128.2, 127.6 (aromatic quarternary C's), 127.1, 126.6, 125.9, 125.8, 124.8, 124.5, 123.4 (aromatic CH's), 74.3 (CH_2), 46.3 (CH); ν_{max} (DCM): 3069 (aromatic CH), 2980 (aliphatic CH), 1776 (carbonate C=O), 1611 (aromatic C=C), 1504, 1142 cm^{-1}; λ_{max}(DCM) 368 (ε 17600 $dm^3mol^{-1}cm^{-1}$), 302 (43000), 290 (33800), 254 nm (66200), m/z (FAB): 460, 458 (M^+); HRMS: 458.10737, $C_{31}H_{19}O_2Cl$ requires 458.10735. Crystals suitable for X-ray diffraction were obtained by slow evaporation of a solution of **(6)** in 1,4-dioxane.

Introduction of the Tbfmoc group onto resin bound polypeptides. As a general method, the Fmoc deprotected resin bound peptide is washed sequentially with DMF and then 1,4-dioxan. The resin is then swollen in 1,4-dioxan and the TbfmocCl **(6)** (3 equiv.) and DIEA (1 equiv.) are added. The reaction mixture is then sonicated in the dark for 1 - 3 hours with occasional mixing before the resin is filtered off, washed with 1,4-dioxan,dichloromethane and dried.

17-Methylenetetrabenzo[*a,c,g,i*]fluorene (8).

A solution of 17-tetrabenzo[*a,c,g,i*]fluorenylmethanol (50 mg, 0.126mmol) and triethylamine (1 ml, 7.17 mmol; 57 equiv.) in dichloromethane (2 ml) was stirred overnight at room temperature. Phosgene (1 ml, 1.93 mole; 15 equiv; 1.93 M in toluene) was then added. A red colouration of the reaction mixture was immediately observed and a gas was released. The reaction mixture was stirred for 5 minutes, then diluted with water (50 ml) and acidified with H_2SO_4 (2M; pH=1). After extraction with dichloromethane (3 x 30 ml), the combined organic phases were washed with water (2 x 50 ml) and dried over $MgSO_4$. The solvent was then removed *in vacuo* to give an orange residue. Purification by silica gel chromatography (toluene) gave the *title compound* as a red crystalline solid (7.3 mg, 15%); m.p. 169-170°C; Found: C, 94.9; H, 4.90. $C_{30}H_{18}$ requires C, 95.2: H, 4.76%[20]; TLC: R_f 0.79 (toluene): δ_H(80 Mhz, $CDCl_3$): 8.82-8.41 (8H, m, aromatic), 7.77-7.51 (8H, m, aromatic), 6.92 (2H, s, CH_2); δ_C(50 Mhz, $CDCl_3$): 147.1, 136.8, 133.4,131.6, 131.1, 128.5, 127.4 (aromatic quarternary C's and quarternary C (alkene)), 127.8, 127.3, 126.3, 125.6, 125.2, 124.7, 123.7, 123.5 (aromatic CH's), 122.8 (CH_2); λ_{max}(DCM) 454 (ε 2700 $dm^3mol^{-1}cm^{-1}$), 328 (44280), 314 (50220), 260 nm (66200), m/z (FAB): 378 (M^+); HRMS: 378.14082, $C_{30}H_{18}$ requires 378.14094.

Coupling of the C-terminal amino acid onto 4-alkoxybenzylalcohol (Wang) resin. A solution of Fmoc amino acid (1 mmol) and N,N'-diisopropylcarbodiimide (DIC) (0.5 mmol) in DMF (20ml) was stirred for 15 minutes at room temperature, then 4-benzyloxybenzylalcohol functionalised polystyrene resin (Wang) (1.0 g, 0.8 mmol) was added, together with a catalytic amount of 4-(N,N'-dimethylamino)-pyridine and the mixture was then sonicated for 1-2 hours. The functionalised resin was then removed by filtration and sequentially washed with DMF, 1,4-dioxan and dichloromethane, before being dried under vacuum. The loading of the functionalised resin was then determined by treating a known weight of resin with 20% piperidine/DMF in a 10ml volumetric flask, for 20 minutes in a sonic bath. The UV absorbance of the supernatant was then measured at 302 nm and the loading calculated using the Beer-Lambert law (ε_{302} = 15400 for fulvene-piperidine adduct). This procedure generally gives a resin with a functionality in the region of 0.1 mmol/g.

α-Calcitonin gene related peptide (8-37). H-Val.Thr.His.Arg.Leu.Ala.Gly.Leu.Leu.Ser.Arg.Ser.Gly.Gly.Val.Val.Lys.Asn.Asn.Phe.Val.Pro.Thr.Asn.Val.Gly.Ser.Lys.Ala.Phe-NH_2. The peptide was synthesised using commercially available tricyclic amide linker functionalised polystyrene[15] (0.38 g, 0.65 mmol/g, 0.25 mmol) and single coupling each amino acid as the corresponding HOBt ester. At the completion of synthesis the resin was filtered and washed with 1,4-dioxane, before being treated with TbfmocCl (**6**) (70 mg, 0.15 mmoles, ~3eq.)and DIEA (9 μL, 0.05 mmoles, ~1 eq.) in 1,4-dioxane (20 ml) for 1 hour in a sonic bath, whilst protected from

daylight. The resin was then removed by filtration and thoroughly washed with 1,4-dioxane, dichloromethane and dried. The dry Tbfmoc functionalised resin bound peptide was then cleaved from the resin by stirring for 3 hours with a mixture of ethane-1,2-dithiol (2 ml), water (0.5 ml), thioanisole (0.5 ml), triisopropylsilane (0.5 ml) and trifluoroacetic acid (10 ml). The TFA was then removed by rapid evaporation under reduced pressure and the crude peptide precipitated with diethyl ether, filtered off and dried. The Tbfmoc-labelled peptide was then affinity purified on porous graphitised carbon (PGC) by adding PGC (1 g, Hypersil HPLC grade) to a stirring solution of the crude peptide in 70% acetonitrile/water. After stirring overnight the PGC was centrifuged and the solvent decanted off. This washing process was then repeated with a mixture of 6M guanidine hydrochloride/isopropanol (1:1) (5 x 50 ml). The desired sequence was then released from the PGC by treatment with 10% piperidine in 6M guanidine hydrochloride/isopropanol (1:1). The PGC was then pelletised by centrifugation and the deprotection solution neutralised with acetic acid before the isopropanol was removed *in vacuo*. The pure calcitonin gene related peptide (8-37) was then isolated by semi-preparative HPLC (Aquapore C4, 100 x 10 mm, 7 μm, A=H_2O (0.1% TFA), B= CH_3CN (0.1% TFA); 5 ml/min. 0-40% B over 30 minutes. λ=229 nm) and lyophilised to give the *title compound* as a white powder (55 mg, 7% yield); amino acid analysis: Asx_3 2.64, Thr_2 1.95, Ser_3 2.73, Pro_1 1.01, Gly_4 4.16, Ala_2 2.15, Val_5 4.63, Leu_3 2.90, Phe_2 1.97, His_1 1.05, Lys_2 2.08, Arg_2 2.13; m/z (laser desorption) 3127.2, $C_{139}H_{231}N_{44}O_{38}$ requires 3126.6; HPLC (Aquapore C4, 100 x 4.6 mm, 7 μm, A=H_2O (0.1% TFA), B= CH_3CN (0.1% TFA); 1 ml/min. 0-90% B over 30 minutes. λ=214nm), R_t=17.5 min., 50%B.

Methylated DNA Binding Domain of MeCP2 (MBD) (MeCP2 78-162). The synthesis was carried out on an initial scale of 0.144mmol using the functionalised resin Fmoc.Arg(Pmc)-($OCH_2C_6H_4OR$) (1.019g, 0.142 mmol/g). All amino acid side chains were protected as described previously with the exception of asparagine and glutamine, which were incorporated without side chain protection. All amino acids (with the exception of glycine) were double coupled, apart from residues 69 to 56 which were treble coupled with the third coupling cycle time extended, and residues 41 to 25 which were allowed to couple for an extended period during the second coupling cycle. The N-terminal Fmoc group was left on at the completion of the assembly phase and the resin bound product was capped for 30 minutes in a sonic bath before being washed sequentially with DMF, 1,4-dioxan, dichloromethane and dried to give 2.82g of resin bound product.

500 mg of the Fmoc protected, resin bound product was treated with 20% piperidine/DMF (10 ml) for 10 minutes in a sonic bath, before the resin bound product was filtered off, washed with DMF, 1,4-dioxan, dichloromethane and dried. This dry resin was then added to a suspension of TbfmocCl (**6**) (230 mg, 0.5 mmol) in dichloromethane (10ml) and DIEA (64 μL, 0.37 mmol) was added before the reaction flask was sealed, covered in aluminium foil and sonicated for three hours with occasional mixing. The resin bound Tbfmoc-protein was then washed thoroughly with dichloromethane, dried and cleaved from the resin by stirring for 4 hours in the dark, with a mixture of TFA (10 ml), phenol (0.75 g), EDT (1.5 ml), thioanisole (0.5 ml) and water (0.5 ml).

The resin was then removed by filtration and the filtrate concentrated *in vacuo* to give an oil, which yielded solid Tbfmoc-protein on trituration with diethyl ether. The solid Tbfmoc-protein was then filtered off, washed with diethyl ether and dissolved in aqueous acetic acid (50%) before being applied to the top of a column of Sephadex G-50 (10 x 800 mm) and eluted with aqueous acetic acid (30%). Pure Tbfmoc-protein was obtained by semi-preparative HPLC (Vydac C18, 250 x 22mm, 10 μm, A=H_2O, B=CH_3CN, 0.1% TFA; 9 ml/min. 10-70% B over 40 min. λ=364 nm) and concentrated to a small volume (5-10ml) by lyophilisation. The Tbfmoc was then deprotected by adding piperidine (1ml) and mixing for 5 minutes, before the solution was cooled on ice, acidified (AcOH), applied to the top of a column of Sephadex G-50 (10 x 800 mm) and eluted with aqueous acetic acid (30%). The protein containing fractions were then pooled and pure MBD protein was obtained after semi-preparative HPLC (Vydac C18, 250 x 22mm, 10 μm, A=H_2O, B=CH_3CN, 0.1% TFA; 9 ml/min. 10-70% B over 40 min. λ=214 nm) and lyophilisation as a white solid (32.8 mg, 2.3% yield from 500 mg sample of crude resin bound product); amino acid analysis (48hr. hydrolysis): Asx_{10} 9.53, Thr_5 4.44, Ser_7 5.80, Glx_6 7.09, Pro_6 5.83, Gly_7 7.27, Ala_4 4.43, Val_4 4.00, Met_1 1.05, Ile_4 3.87, Leu_5 5.26, Tyr_4 4.12, Phe_4 4.09, Lys_8 8.08, Arg_9 9.16, Trp_1 (N/A); m/z (laser desorption) 9727, $C_{431}H_{683}N_{126}O_{129}S_1$ requires 9726.0; HPLC (Vydac C18, 250 x 4.6mm, 5 μm, A=H_2O, B=CH_3CN, 0.1% TFA; 1 ml/min. 10-70% B over 45 min. λ=214 nm), Rt= 24.0 min., 42.5%B. In addition, the first twenty six amino acid residues were sequenced by Edman degradation and were found to be in agreement with the required sequence. Purification of MBD using method A, as described for α-CGRP, gave material identical to that obtained from the above route (method B).

Restriction Alleviation Protein. The synthesis was carried out on a 0.25 mM scale using Fmoc-alanine functionalised 4-alkoxybenzylalcohol resin (400 mg, 0.614 mmol/g). All amino acid side chains were protected as described previously, with the exception of asparagine, glutamine and cysteine which were incorporated as the corresponding triphenylmethyl (trityl) derivatives. All amino acids (with the exception of glycine) were double coupled, with the regions 54-45 and 30-1 being coupled for an extended period of time during the second coupling cycle. At the end of the synthesis the Fmoc was left on the N-terminal methionine, and the resin bound product was capped for 30 minutes in a sonic bath. The resin was then washed with DMF, 1,4-dioxan, dichloromethane and dried to give 2.18 g of Fmoc protected resin bound protein.

The resin was then treated with 20% piperidine/DMF for 15 minutes in a sonic bath before being filtered off, washed with DMF, 1,4-dioxan, dichloromethane and dried. The dry resin bound protein was then added to a suspension of TbfmocCl (400 mg, 0.88 mmol) in dichloromethane (20 ml) and DIEA (60 μl, 0.34 mmol) was added before the reaction flask was sealed, covered in aluminium foil and then sonicated for 3 hours with occasional mixing. The Tbfmoc-peptide-resin was then filtered off, washed thoroughly with dichloromethane and dried.

A 500mg portion of the dry Tbfmoc resin bound protein was then added to a mixture of EDT (2 ml), thioanisole (0.5 ml), phenol (0.75 g), water (0.5 ml), TIS (0.5 ml) and stirred for 15 minutes, before TFA (10 ml) was added and the mixture stirred for a further 4 hours whilst protected from daylight. The resin was then removed by filtration and the filtrate concentrated *in vacuo* to give an oil, which yielded solid Tbfmoc-protein on trituration with diethyl ether. The solid Tbfmoc-protein was then filtered off, washed with diethyl ether and dissolved in aqueous acetic acid (50%) before being applied to the top of a column of Sephadex G-50 (10 x 800 mm)and eluted with aqueous acetic acid (30%). Pure Tbfmoc-protein was obtained by semi-preparative HPLC (Vydac C18, 250 x 22 mm, 10 μm, A=H_2O, B=CH_3CN, 0.1% TFA; 9 ml/min. 30-90% B over 25 min. λ=364 nm) and lyophilised to give 140 mg white solid. The Tbfmoc was then deprotected under reducing conditions (6M Gdm.HCl, pH 8.5 Tris, 0.1M, containing excess dithiothreitol) for 4 hours at 37°C before being desalted (Sephadex G-50, 30% AcOH) and purified by HPLC to give 40 mg of fully reduced Ral protein after lyophilisation; Asx_7 5.11, Thr_5 4.74, Ser_1 1.07, Glx_9 8.08, Pro_2 2.02, Gly_4 4.37, Ala_5 5.52, Cys_4 0.99, Val_3 3.17, Met_5 4.48, Ile_3 3.12, Leu_2 2.31, Tyr_1 0.71, Phe_2 2.10, Lys_8 8.17, Arg_3 3.11, Trp_2 (N/A); m/z (laser desorption) 7607.3, $C_{326}H_{526}N_{93}O_{98}S_9$ requires 7604.49; HPLC (Vydac C18, 250 x 4.6 mm, 5 μm, A=H_2O, B=CH_3CN, 0.1% TFA; 1 ml/min. 10-90% B over 30 min. λ=214 nm), Rt= 17 min., 56%B.

Acknowledgements. We thank Applied Biosystems Inc., Rhone-Poulenc Rorer and the S.E.R.C. Molecular Recognition Initiative for support for S.L.I., G.R. and A.R.B. respectively, and Alexander J. Blake, Magnus Brisander and Simon Parsons for crystallographic data. In addition we thank Professor A. Bird and Dr X. Nan for carrying out gel retardation assays on synthetic MBD and Mr K.T. Shaw and Mr B. Whigham for technical support. We thank Professor J.H. Knox for constant encouragement.

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11. Crystal Data:- $C_{31}H_{19}ClO_2$, 0.53 x 0.27 x 0.19 mm, M = 458.91, monoclinic, space group $P2_1/c$, a = 10.658(5), b = 10.911(7), c = 18.254(10) Å, β = 91.53(4)°, V = 2122 Å^3, Z = 4, D_c 1.436 Mg m^{-3}, T= 150K, μ = 0.209 mm^{-1}, F(000) = 952.

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20. In this and related studies microanalysis results on tetrabenzo[*a,c,g,i*]fluorene derivatives have proved generally unsatisfactory, giving lower carbon content than that calculated for the expected molecular formulae. This has been attributed to the large number of quarternary carbons present leading to poor combustion. Increased oxygen concentrations and the addition of vanadium pentoxide have still not proved entirely satisfactory and this is the subject of on-going investigations in this laboratory.

(*Received in UK* 14 *July* 1995; *revised* 4 *September* 1995; *accepted* 8 *September* 1995)