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Introduction of foreign DNA into mycobacteria using a shuttle phasmid

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Mycobacteria are major pathogens of man and animals. There are ~10 million cases of tuberculosis world wide with an annual mortality of three million[1] people. Leprosy, caused by *Mycobacterium leprae*, afflicts over ten million people, primarily in developing countries[2]. *M. tuberculosis* and mycobacteria of the *M. avium-intracellulare-scrofulaceum* (MAIS) group are major opportunistic pathogens of patients with acquired immune deficiency syndrome (AIDS)[3]. *M. paratuberculosis* is the cause of Jöhne's disease in cattle. Yet, BCG (Bacille Calmette-Guerin), an avirulent strain of *M. bovis*, is the most widely used human vaccine in the world, having been administered to about 2.5×10^9 people since 1948 (ref. 4). BCG was highly protective against tuberculosis in England[5], but has been found not to be effective in preventing pulmonary tuberculosis in adults in Southern India[6]. We have initiated studies to develop the methodology for efficient gene transfer in mycobacteria. We have constructed recombinant shuttle phasmids which are chimaeras containing mycobacteriophage DNA into which an *E. coli* cosmid is inserted. They can replicate in *E. coli* as plasmids and in mycobacteria as phages, and transfer DNA across both genera. These shuttle vectors permit for the first time the introduction of foreign DNA by infection into *M. smegmatis* and BCG. By introducing and ultimately expressing genes for protective antigens for a variety of pathogens, it may be possible to develop cultivatable mycobacteria into useful multivaccine vehicles.

To develop a system for the manipulation of DNA in mycobacteria it was first necessary to devise an efficient means of transferring DNA into the bacillus. We adapted the technology for preparing protoplasts developed for the related actinomycete, *Streptomyces*[7,8] for use with *M. smegmatis*, with the addition of polyethylene glycol to facilitate entry of DNA molecules into bacterial protoplasts[9]. The system chosen to evaluate optimum conditions for DNA transfer into mycobacteria was the transfection of DNA from lytic mycobacteriophages, because the results obtained were quantitative and readily apparent within 24 h. Transfection experiments were initiated with DNA from mycobacteriophage D29, which propagates on a wide variety of mycobacteria and forms large clear plaques on *M. smegmatis*. Restriction analyses of ligated and unligated D29 DNA demonstrated that the phage genomic DNA was double stranded, ~50 kilobases (kb) in size, and possessed cohesive ends (data not shown).

The results of transfection of *M. smegmatis* spheroplasts by mycobacteriophage D29 DNA are illustrated in Fig. 1. Efficiencies of 10^3-10^4 plaque-forming units (PFU) per μg D29 DNA were routinely obtained. These plaques were shown to be the result of transfection of *M. smegmatis* spheroplasts by three criteria: first, transfection was abolished by DNase; second, osmotic shock of treated cells prevented productive transfection and third, spheroplasts derived from a D29 phage-resistant mutant of *M. smegmatis* were transfected at frequencies comparable to those of the parent strain.

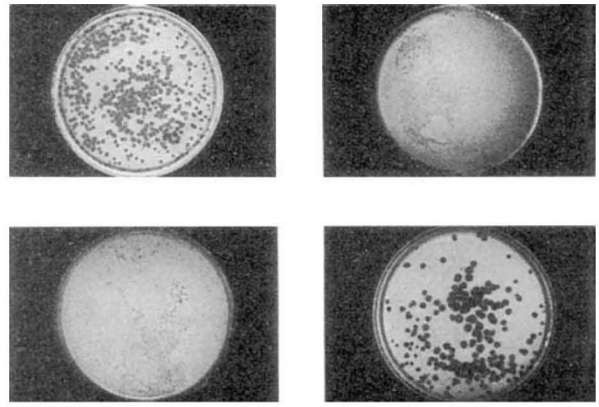

Fig. 1 Transfection of *M. smegmatis* spheroplasts with mycobacteriophage D29 DNA. Top left, *M. smegmatis* spheroplasts transfected with D29 DNA; top right, same but D29 DNA treated with DNAse; bottom left, same as top left but plated on medium lacking sucrose. Bottom right, spheroplasts from D29-resistant *M. smegmatis* transfected with D29 DNA.

Methods. Spheroplasts were prepared as described below from the *M. smegmatis* strain mc^26, a single colony isolate which is the predominant colony type isolated from the ATCC 607 *M. smegmatis* stock culture and which forms orange rough colonies on regeneration media[20]. A second strain, mc^211, was isolated as a spontaneous D29-resistant isolate of the ATCC 607 *M. smegmatis* stock culture when 10^8 cells were mixed with 3×10^8 PFU of D29 and plated on tryptic soy agar plates. D29-resistant colonies arose at a frequency of 10^{-7}. Spheroplasts of mc^26 were mixed with 1 μg of D29 DNA, and subsequently one tenth of that mixture was plated on tryptic soy agar plates with or without 0.5 M sucrose and then overlayed with the appropriate soft agar containing 10^8 mc^26 cells. The DNase treatment was performed by adding DNase I (sigma) at final concentration of 50 μg ml^{-1} to the D29 DNA. Equivalent amounts of mc^211 spheroplasts were used in the experiment above, but then subsequently overlaid with mc^26 cells to assay plaque forming units. Preparation of mycobacteriophage and phage DNA: Plate lysates of D29 (ref. 21) were prepared using mc^26 cells and tryptic soy agar as described previously[22]. Phage were purified on two CsCl equilibrium gradients followed by extensive dialysis against MP buffer (10 mM Tris, *p*H 7.6, 100 mM NaCl, 10 mM $MgSO_4$, 2 mM $CaCl_2$). DNA was purified from phage by proteinase-K treatment, phenol-chloroform extraction and extensive dialysis against TE buffer. Spheroplasts of *M. smegmatis* were prepared as for *Streptomyces*[20] using media for the preparation of lysozyme-sensitive cells as for *M. smegmatis*[23]. Briefly, 40 ml of mc^26 cells were grown in tryptic soy broth containing 0.2% Tween-80 at 37 °C to an A_{600} of 0.2, at which time glycine was added to a final concentration of 1%. The cells were incubated for an additional 16 h, harvested by centrifugation, washed twice with 10.3% sucrose, and resuspended in P (protoplast) buffer[20] containing 2 mg ml^{-1} lysozyme. After a 2-h incubation at 37 °C, the spheroplasts were pelleted and resuspended in 10 ml P buffer. For each transfection, 2.5 ml of the spheroplast suspension was pelleted, the supernatant fluid was carefully decanted and the spheroplasts were resuspended in the remaining drop of P buffer. Following the addition of 1 μg of DNA, 0.5 ml of 25% polyethylene glycol (PEG-1000) prepared in P buffer was mixed with the spheroplasts and DNA. This mixture was diluted with 5 ml P buffer, and the spheroplasts were pelleted and resuspended in 1 ml of P buffer. Samples were transferred to tryptic soy agar with 0.5 M sucrose. The plates were then overlaid with 3.0 ml of soft tryptic soy-sucrose agar and incubated at 37 °C. The plaques were counted after 24 h incubation.

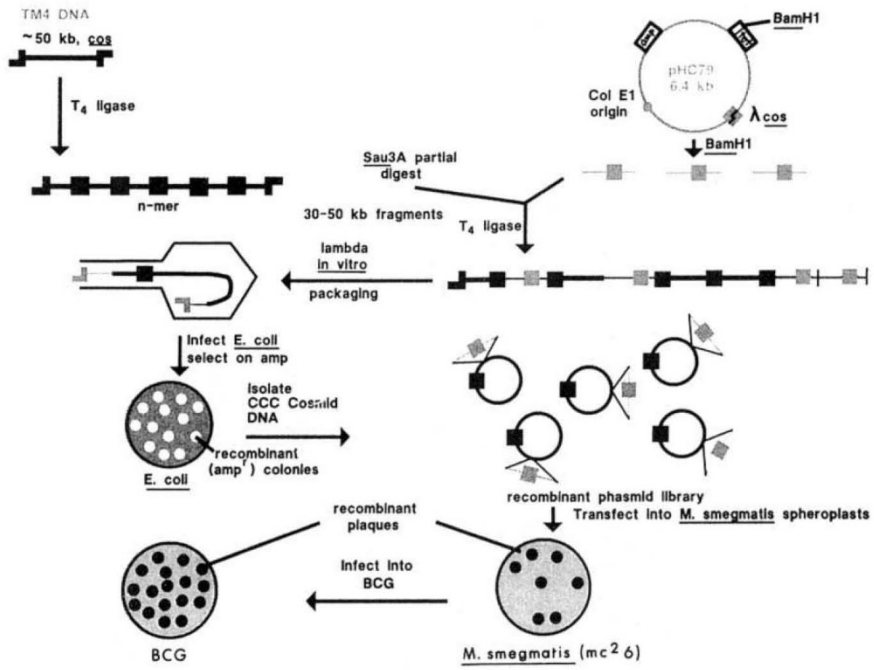

Fig. 2 Diagram of the construction of the shuttle phasmids. TM4 phage DNA was ligated at a concentration of 250 μg ml^{-1} and then partially digested with *Sau*3A to obtain fragments that averaged 30–50 kb. These fragments were ligated at a 1:2 molar ratio of TM4 fragments to pHC79 that had been cleaved with *Bam*HI and alkaline phosphatased. The packaging of an aliquot of this ligation with *in vitro* packaging mix (Gigapack Plus, Stratagene) and subsequent transduction into ER1381 (*hsd*R *mcr*A$^+$ *mcr*B$^+$, E. Raleigh, personal communication), yielded 10^6 ampicillin colonies per μg of TM4 DNA insert, when plated on L-agar containing ampicillin at 50 μg ml^{-1}. A pool of 40,000 ampicillin-resistant clones was prepared by homogenizing colonies in L-broth from the selection plates with a glass spreader. Plasmid was isolated from the pool of clones by alkaline-SDS treatment[24] followed by phenol-chloroform extraction and concentration with ethanol. Covalently closed circular plasmid DNA was transfected into mc^{2}6 spheroplasts as in Fig. 1. The plaques were screened for the presence of pHC79 by performing plaque lifts[25] and Biotrans nylon membranes (ICN Radiochemicals). The membranes were hybridized with pHC79 DNA nick-translated with ^{32}P-dCTP, and autoradiography was carried out.

Our immediate aim was to develop a vector that would permit both the manipulation and amplification of mycobacterial DNA constructs in *E. coli*, and subsequent transfer and replication in a variety of mycobacteria. In particular, we wanted to be able to introduce DNA into fast-growing non-pathogens such as *M. smegmatis*, but also into slow-growing mycobacteria, such as BCG and *M. tuberculosis*. Although plasmids have been found in some mycobacterial strains in the MAIS complex and *M. fortuitum*[10–13], none have yet been described which replicate in *M. smegmatis*, BCG, or *M. tuberculosis* and, with one exception, none possess selectable markers. In contrast, a variety of phages that replicate in *M. smegmatis*, BCG, and *M. tuberculosis* have been described and long used for typing isolates. We reasoned that the best strategy might be to construct a vector that would replicate as a plasmid in *E. coli* and as a phage in mycobacteria. A λ-ColE1 vector with dual properties in *E. coli* was previously designated a phasmid[14]. Our aim was to develop bifunctional vectors which were similar to those described for *Streptomyces*[15], except that they could also be packaged *in vitro* into λ phage, which would permit highly efficient transfer of DNA into *E. coli* and subsequently into mycobacteria. For this purpose we selected the mycobacteriophage TM4, a temperate phage isolated from *M. avium*[16], which we had found replicates in *M. smegmatis*, BCG, and *M. tuberculosis*. This phage has a double-stranded DNA genome of ~50 kilobases (kb) and possesses cohesive ends (Fig. 3).

Our strategy of using cosmid cloning[17] to introduce an *E. coli* plasmid replicon into a non-essential region of phage TM4 is illustrated in Fig. 2. The cohesive ends of TM4 genomes were ligated together to form long concatamers which were then partially digested with *Sau*3A to generate a set of DNA fragments that contained the entire TM4 genome or genomes with small deletions, but had been cleaved at any of the *Sau*3A sites in the genome. These DNA fragments were then ligated to the cosmid pHC79 (ref. 18) which had been cleaved with *Bam*HI. To select for recombinant molecules of the appropriate size, the ligation mixture was packaged into phage λ heads *in vitro*. The resulting phage particles were transduced into *E. coli* and clones containing recombinant pHC79::TM4 DNA molecules were selected on media containing ampicillin. Covalently closed circular plasmid DNA was isolated from 40,000 pooled ampicillin-resistant colonies. This library, containing recombinant

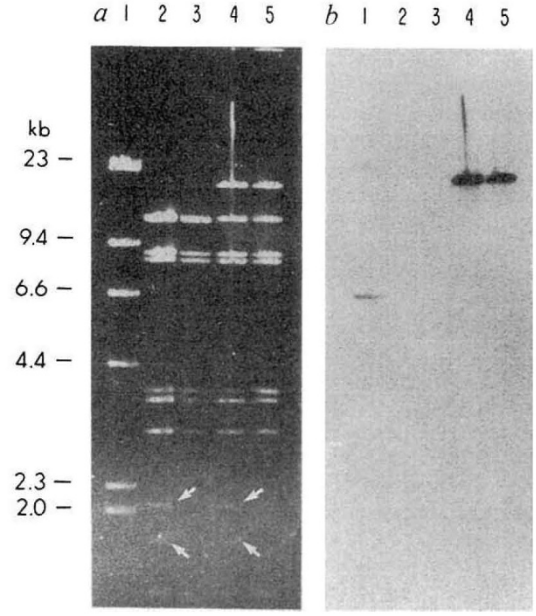

Fig. 3 *a*, Ethidium bromide stained 0.7% agarose gel of TM4 and phAE1 DNAs digested with *Kpn*I. Lane 1, λ DNA digested with *Hin*dIII. Lanes 2 and 3, TM4 DNA that was unligated (lane 2) or ligated (lane 3) before digestion, cut with KpnI. Lanes 4 and 5, phAE1 DNA that was isolated from phage particles propagated on *M. smegmatis* (lane 4) and phAE1 that had been isolated from *E. coli* cells as a plasmid (lane 5). Arrows, unannealed 2.1- and 1.8-kb TM4 cohesive end fragments, present in DNA preparations from mycobacteriophage. These bands are absent when phage DNAs are ligated before digestion or when shuttle phasmid DNA is isolated as a plasmid. *b*, Southern blot analysis of phAE1 using pHC79 as a probe. An autoradiograph of *a*, after performing a Southern blot[26] onto nylon membrane and probing with pHC79 DNA that had been nick-translated with ^{32}P-dCTP.

molecules of TM4 genomes into which pHC79 cosmid DNA had been randomly inserted in *Sau*3A sites around the TM4 genome, was transfected into *M. smegmatis* spheroplasts to select for TM4 phages which had pHC79 inserted in non-essential regions. The transfection yielded 100 PFU μg^{-1} of plasmid DNA. Among 4,000 plaques screened for the presence of

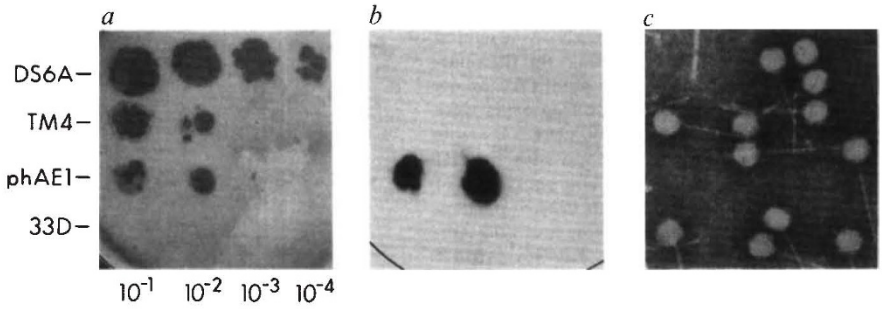

Fig. 4 Replication of phAE1 on BCG and ultrastructural analysis of phAE1 mycobacteriophage particles. *a*, Comparison of lysis of the Glaxo vaccine strain of BCG by DS6A (ref. 22), a mycobacteriophage known to form plaques on *M. tuberculosis* and BCG, but not other mycobacteria; phage 33D, known to form plaques on *M. tuberculosis* and not BCG[27]; and phages TM4 and phAE1. Titres of phage (PFU ml^{-1}) used at 10^{-1} dilution were: DS6A, 2×10^6 on *M. tuberculosis* strain H37Ra; 33D, 2×10^6 on *M. smegmatis* strain mc^26; TM4, 3×10^8 on mc^26; and phAE1, 3×10^8 on mc^26. Dilutions of phages (5 μl) were spotted on a soft agar overlay containing 10^8 BCG cells prepared as described by Jones *et al.*[28] and the resulting lysis was photographed after incubation for 10 d at 37 °C. Similar results were obtained for the Danish and Pasteur strains of BCG. Both TM4 and phAE1 form plaques 5–6 logs of ten less efficiently on BCG than on mc^26. This decrease is most probably a result of restriction of phage grown on *M. smegmatis* by BCG, because phage prepared from BCG phage lysates give equal efficiencies when plaqued on BCG and mc^26. *b*, Presence of pHC79 in phAE1 replicated on BCG. A plaque lift was performed on the plate in *a* as described above, hybridized with ^{32}P-labelled pHC79 DNA, and the resulting autoradiograph is shown. *c*, Electron micrograph of shuttle phasmid phAE1 mycobacteriophage particles. CsCl-purified phage particles were placed on carbon coated, Parloidon-coated grids, blotted and washed with one drop of 1% phosphotungstic acid. Electron micrographs were taken using a JEOL 1200EX electron microscope at 80 kV, magnification ×30,000.

pHC79, only 10 were positive. Following plaque purification and propagation on *M. smegmatis* cells, one such phage (phasmid phAE 1) was studied in more detail. Restriction fragment analysis of DNAs isolated from a number of independent phages that had resulted from the transfection of the pHC79::TM4 DNA library into *M. smegmatis*, and that did not hybridize with pHC79, revealed them to be identical to TM4 DNA. We believe these phages resulted from a recombination event occurring in transfected cells containing two or more pHC79::TM4 molecules, yielding a wild-type TM4 genome.

DNA isolated from phAE1 phage particles grown on *M. smegmatis* were subsequently ligated at the TM4 cohesive ends and packaged *in vitro* into bacteriophage λ heads using the λ cohesive ends, yielding particles which efficiently transduced ampicillin resistance to *E. coli* cells. The ability of these shuttle phasmids to use the λ *in vitro* packaging system will greatly facilitate subsequent cloning of genes into these vectors. Gel analysis of TM4 and phAE1 DNAs digested with *Kpn*I revealed the presence of a new 18-kb fragment in phAE1 with a subsequent loss of one of the 12-kb doublets in TM4 (Fig. 3*a*). Southern analysis confirms that the cosmid pHC79 is present in the 18-kb fragment (Fig. 3*b*), indicating that pHC79 has been inserted in a non-essential region of a 12-kb *Kpn*I fragment of the TM4 genome. The phAE1 DNAs, isolated from mycobacteriophage particles or plasmid DNA from *E. coli*, showed identical restriction patterns, except for the presence of the unannealed TM4 cohesive ends in the phage DNA (Fig. 3*a*). The phAE1 mycobacteriophage particles have hexagonal heads that average 50 μm in diameter and long tails of 180–220 μm in length with a disk-like baseplate present on many of the tails (Fig. 4*c*). This structure is very similar to the parent TM4 phage[14].

One of the goals of this research is to enable us to introduce DNA encoding important antigens into BCG vaccine strains. However, transformation of slow-growing mycobacterial strains, including BCG, has not yet been achieved. It is therefore gratifying that the shuttle phasmid phAE1 obtained from *M. smegmatis*, like its parent TM4, is able to infect, and replicate in, three different *M. bovis*-BCG vaccine strains tested, the Glaxo, Pasteur and Danish BCGs (Fig. 4*a* and *b*). Current efforts are directed towards developing conditions for stable expression of cloned genes in mycobacterial cells.

We believe that the ability to infect BCG vaccine strains with the shuttle phasmids provides a novel means of introducing cloned genes into slow-growing mycobacteria and offers the possibility of developing recombinant mycobacterial multivaccine vehicles. This could be achieved if protective antigens from a variety of pathogens, particularly those requiring T-cell memory or effector function, could be introduced and stably expressed. BCG vaccine has been administered to billions of people over the past 35 years with extraordinarily few side-effects and a mortality rate of 60 per billion of those treated[19]. BCG is probably the most potent immunological adjuvant known, especially for engendering cell-mediated immunity. It is the only vaccine currently given at birth and a single inoculation induces cell-mediated immunity for 5–50 years, at a cost of only $0.055 per inoculation. We hope as well that the methodology described here will facilitate the development of a molecular genetic system in pathogenic mycobacteria that will permit better understanding, and perhaps molecular modification, of their pathogenicity.

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Optically active benzo[*c*]phenanthrene diol epoxides bind extensively to adenine in DNA

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Reactions of diol epoxide metabolites of carcinogenic polycyclic aromatic hydrocarbons with DNA are thought to initiate the carcinogenic process[1,2]. Although formation of a benzo[*a*]pyrene (BaP) diol epoxide–deoxyguanosine adduct has been held responsible for biological activity[3–5], the more potent carcinogen, 7,12-dimethylbenz[*a*]anthracene (DMBA) binds extensively to deoxyadenosine residues in DNA, suggesting that hydrocarbon carcinogen-deoxyadenosine adducts may be instrumental in tumour initiation[6–8]. Because the bay region diol epoxides[9] of benzo[*c*]phenanthrene (BcPh) are very active tumour initiators[10], and the relative activities of the four configurationally isomeric 3,4-diol 1,2-epoxides (Fig. 1) are known[11], we examined their reactions with DNA. Each BcPh diol epoxide isomer exhibits a remarkable preference for covalent binding to DNA over hydrolysis, each yields a unique distribution of products with the nucleosides of DNA and each reacts extensively with deoxyadenosine residues in DNA. The relative tumour initiating activities of these stereoisomers is best reflected by the relative yields of one of the deoxyadenosine adducts formed.

Tetraol formation in the presence of DNA indicated that 60–75% of the four BcPh diol epoxides bound covalently to DNA[12] in contrast to just 6% for a BaP diol epoxide[12,13]. DNA adduct formation has now been measured directly for one of the BcPh diol epoxide isomers. When calf thymus DNA (0.8 mg ml^{-1}) was treated with (−)-diol epoxide-2 (Fig. 1) at 0.097, 0.020 and 0.004 mg ml^{-1}, 61%, 67% and 67% respectively of the diol epoxide was recovered as nucleoside adducts, in agreement with estimates from tetraol formation. This remarkable reactivity towards DNA could contribute to the high tumorigenic activity of some of the BcPh diol epoxides but cannot be the predominant factor, because all four stereoisomers react extensively with DNA whereas their tumour-initiating activities range from low to high[11].

Chromatographic analyses indicated that at least four different diol epoxide-nucleoside adducts were formed from each isomer. The nucleoside of origin of the principal adducts (Fig. 1) was established by comparing their spectral and chromatographic properties with those of adducts formed in separate reactions of each diol epoxide with individual nucleotides. The UV spectra of the deoxyadenosine and deoxyguanosine adducts were particularly useful for initial structural assignments. Only 8 of the 16 spectra (Fig. 2) are shown (solid lines for adducts with a 1 subscript in Fig. 1 and broken lines for adducts with a 2 subscript) because the spectra of the four adducts derived from the (+)-enantiomer of each diol epoxide ($dGuo_1$, $dGuo_2$, $dAdo_1$ and $dAdo_2$, see Fig. 1 for nomenclature)

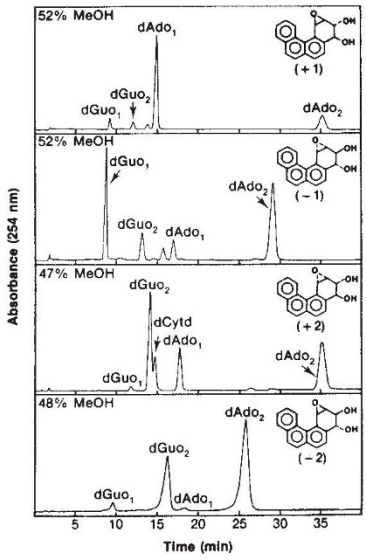

Fig. 1 Chromatographic separations of adducts formed in the reactions of configurationally isomeric BcPh 3,4-diol-1,2-epoxides with DNA. For diol epoxide-1, the benzylic hydroxyl group and the eposide oxygen are *cis* and for diol epoxide-2, these groups are *trans*. To aliquots (0.5 ml) of a calf thymus DNA (Sigma) solution (0. 8 mg ml^{-1} in 0.05 M Tris-HCl buffer, *p*H 7) was added 0.05 ml of an acetone solution of the appropriate isomeric diol epoxide (2 mg ml^{-1}). After 5 h at 37 °C, each reaction mixture was diluted to 1 ml with 0.05 M Tris-HCl and was then extracted three times with 2 vol ethyl acetate and once with ether. Residual ether was removed from the aqueous solution in a stream of N_2. To convert the DNA to deoxyribonucleoside, each sample was incubated with deoxyribonuclease, snake venom phosphodiesterase and alkaline phosphatase[6]. The digest was then loaded on a Waters Sep-pak C_{18} cartridge which was washed with water (20 ml) and 25% methanol (20 ml) before elution of the benzo[*c*]phenanthrene diol epoxide/deoxyribonucleoside adducts in methanol (2.6 ml). The components of the methanol eluates were then eluted isocratically in the aqueous methanol solvents indicated in Fig. 1 from an Altex Ultrasphere ODS column[6]. The adducts are labelled as deoxyguanosine (dGuo), deoxyadenosine (dAdo), or deoxycytidine (dCytd) adducts by comparison of retention times and UV absorption spectra with those for products from reactions of individual nucleotides (0.5 ml at 4.0 mg ml^{-1} in 0.01 M Tris-HCl buffer, *p*H 7) with diol epoxide solution (0.05 ml at 2 mg ml^{-1} in acetone). These reaction mixtures were extracted with organic solvents, the *p*H was raised to 9.0 and alkaline phosphatase (25 units ml^{-1}) was added before incubation overnight. Products were recovered on Sep-paks, as for the DNA reactions. Under the conditions used here, retention times in minutes with percentage of total products given in parenthesis for products $dGuo_1$, $dGuo_2$, $dAdo_1$ and $dAdo_2$, respectively were: (+)-diol epoxide-1, 9.5 (5.5%), 13.0 (5%), 14.9 (67%) and 36.1 (21%); (−)-diol epoxide-1, 8.4 (28%), 13.1 (10.5%), 16.3 (7%) and 28.2 (49%); (+)-diol epoxide-2, 11.5 (1.5%), 13.5 (35%), 16.3 (16.5%) and 32.1 (34.5%); (−)-diol epoxide-2, 10.4 (3%), 17.4 (30%), 19.6 (1.5%) and 27.6 (64%). As described elsewhere[14], for diol epoxide-1 adducts $dGuo_1$ and $dAdo_1$ and diol epoxide-2 adducts $dGuo_2$ and $dAdo_2$, the epoxide ring has opened to yield *trans* products whereas for diol epoxide-1 adducts $dGuo_2$ and $dAdo_2$ and diol epoxide-2 adducts $dGuo_1$ and $dAdo_1$, the epoxide ring has opened to yield *cis* products.

can be superimposed on the spectra of the same adducts derived from the (−)-enantiomer. The members of each pair of adducts with identical spectra presumably differ from one another only in that there is a mirror-image relationship between the chiral centres on the BcPh residues in each case. The spectra are useful also for distinguishing deoxyadenosine adducts from deoxyguanosine adducts and for distinguishing the two isomeric products formed with each purine nucleoside by a given diol epoxide (Fig. 2).