Kinetics of DNA adduct formation and removal in mouse hepatocytes following in vivo exposure to 5,9-dimethyl dibenzo[c,g]carbazole

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5,9-Dimethyl dibenzo[c,g] carbazole (DMDBC), a potent mouse hepatocarcinogen, has been shown to induce a non-linear increase in mutant frequency in the liver of the transgenic Muta™Mouse. To gain insight into the mechanisms underlying the mutagenicity of DMDBC in vivo, DNA damage formation and removal were monitored in mouse hepatocytes over 4–144 h after a single skin application of 10 or 90 mg/kg DMDBC. DNA adducts were measured by 32P-post-labeling. DNA repair was assessed by: (i) the unscheduled DNA synthesis (UDS) assay, which measures [3H]thymidine incorporation into hepatocyte DNA undergoing excision repair; (ii) the Comet assay, which detects DNA strand breaks transiently produced between the incision and rejoining steps of the excision repair process. A plateau of ~400 DNA adducts/10^8 nucleotides was reached 24 h after treatment with 10 mg/kg and remained unchanged until 144 h. UDS activity was significantly induced at 15 and 24 h, while no DNA strand breaks were observed at any sampling time. These results suggest that DNA repair mechanisms were efficiently induced and the formation of a high degree of DNA damage was avoided at this dose level. Following exposure to 90 mg/kg DMDBC, the number of DNA adducts increased sharply to a maximum at 24 h (~8000/10^8 nucleotides) and then declined to ~500/10^8 nucleotides at 144 h. UDS activity was markedly induced from 15 to 72 h. Low levels of DNA strand breaks were observed at 24 and 48 h. The formation of large numbers of DNA adducts and the emergence of DNA strand breaks despite a strong initial induction of UDS activity suggested that DNA repair mechanisms were saturated at this dose level. This phenomenon could partly account for the non-linear induction of gene mutations previously reported in the liver of the transgenic Muta™Mouse.

Introduction

The heterocyclic aromatic hydrocarbon 7H-dibenzo[c,g] carbazole (DBC) is a potent environmental carcinogen. Following s.c. injection and skin application, it induced tumors at the site of administration, as well as in the liver (1–3). To gain knowledge of the DBC mechanism of action, methylated derivatives were synthetized. Among them, 5,9-dimethyl dibenzo[c,g]carbazole (DMDBC) showed a specific tropism for the liver. In this target organ, DMDBC induced DNA adducts, gene mutations and tumors following s.c. injection or skin application (4–6). Interestingly, the mutant frequency (MF), measured in the transgenic Muta™Mouse liver, increased in a non-linear dose-dependent manner. A 4-fold increase in MF was observed 28 days after skin application of 10 mg/kg, compared with a 50-fold increase with 90 mg/kg (6). It was subsequently shown that the marked increase in MF in mice treated with 90 mg/kg likely resulted from induction of liver necrosis and compensatory cell proliferation, which fixed persistent DNA adducts into stable mutations (7).

As it is well known that the initial amount of primary DNA damage, as well as the rates and efficiencies of DNA repair mechanisms, also influence the mutagenic potential of a chemical, these parameters were examined to gain an insight into the mechanisms underlying the non-linear mutagenicity of DMDBC in vivo (8,9). The induction and removal of DNA adducts were monitored in mouse hepatocytes over 4–144 h after a single skin application of 10 or 90 mg/kg DMDBC. DNA adducts were measured using 32P-post-labeling analysis and DNA repair in individual cells was assessed by means of: (i) the unscheduled DNA synthesis (UDS) assay, which measures nucleotide excision repair (NER) of bulky DNA adducts through incorporation of [3H]thymidine during DNA resynthesis (10); (ii) the Comet or the single cell gel electrophoresis assay, which detects a wide range of DNA damage, including DNA strand breaks transiently produced between the initial incision and the terminal rejoining steps of the excision repair process (11,12).

Materials and methods

Animals and treatment

As the transgene is neutral and does not interfere with the physiology of the animal, this study, in which MF was not analyzed, was conducted on CD2F1 mice (13). This strain, which results from the same crossing as the Muta™Mouse, increased in the transgenic liver, and is very widely used to apply the results of this study with those previously obtained in the transgenic mice.

Experiments were conducted with 7- to 9-week-old male CD2F1 mice (Charles River, Saint-Aubin-Lès-Elbeuf, France). The animals were housed in plastic cages under standard conditions (20 ± 2°C, 50 ± 10% relative humidity, 12 h light/dark cycle) and were allowed to acclimatize for 7 days prior to treatment.

Experimental design

An area of ~10 cm^2 was shaved on the animals’ backs with an electric clipper 2 days before treatment. DMDBC was synthesized as previously described (14), dissolved in acetone immediately before administration and applied in a volume of 200 µl. Treated animals received a single skin application of DMDBC at 10 or 90 mg/kg, i.e. 0.84 or 7.62 µmol/mouse. The negative control group received the vehicle alone. As a positive control for the UDS and Comet assays, animals received a single oral administration of 2.5 or 10 mg/kg dimethylnitrosamine (DMN) in water in a volume of 10 ml/kg.

Hepatocyte isolation

At various times after treatment (4–144 h), animals were anesthetized with 150 mg/kg pentobarbital in aqueous solution (Imalgène 1000; Rhône-Mérieux, France). Animals were killed by decapitation, and the liver was excised and rinsed in PBS (pH 7.4). Hepatocytes were isolated by collagenase digestion and cell viability was assessed by trypan blue exclusion. Hepatocytes were distributed in 96-well plates at a density of 5 × 10^4 cells per well and incubated at 37°C in 5% CO2 for 2 h prior to treatment.

Abbreviations: DBC, 7H-dibenzo[c,g]carbazole; DMDBC, 5,9-dimethyl dibenzo[c,g]carbazole; DMN, dimethylnitrosamine; FBS, fetal bovine serum; MF, mutant frequency; NER, nucleotide excision repair; NNG, net nuclear grain; TM, tail moment; UDS, unscheduled DNA synthesis; WME, William’s medium E.
Table I. Kinetics of DNA adduct formation in mouse hepatocytes following skin application of DMDBC

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Animal</th>
<th>Sampling time (h)</th>
<th>4</th>
<th>15</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>144</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1</td>
<td>128</td>
<td>559</td>
<td>299</td>
<td>312</td>
<td>514</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>220</td>
<td>374</td>
<td>336</td>
<td>446</td>
<td>533</td>
<td>666</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>150</td>
<td>344</td>
<td>374</td>
<td>477</td>
<td>364</td>
<td>440</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>166 ± 48</td>
<td>343 ± 31</td>
<td>423 ± 119</td>
<td>407 ± 95</td>
<td>403 ± 95</td>
<td>540 ± 115</td>
<td></td>
</tr>
</tbody>
</table>

Numbers of DNA adducts/10^8 nucleotides of individuals, as well as group means (±SD) are given.

Table II. Kinetics of UDS in mouse hepatocytes following skin application of DMDBC

<table>
<thead>
<tr>
<th>Sampling time (h)</th>
<th>Treatment (mg/kg)</th>
<th>No. of animals</th>
<th>Nuclear grain</th>
<th>Cytoplasmic grain</th>
<th>Net nuclear grain</th>
<th>Cells undergoing repair (%)</th>
<th>S phase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>DMN 2.5</td>
<td>5</td>
<td>42.8 ± 6.6</td>
<td>13.2 ± 2.1</td>
<td>29.6 ± 7.8</td>
<td>77.8 ± 8.2</td>
<td>nd</td>
</tr>
<tr>
<td>15</td>
<td>DMN 10</td>
<td>8</td>
<td>90.7 ± 16.8</td>
<td>14.4 ± 2.6</td>
<td>76.3 ± 17.3</td>
<td>95.7 ± 5.4</td>
<td>nd</td>
</tr>
<tr>
<td>Acetone 3</td>
<td>DMDBC 10</td>
<td>3</td>
<td>8.4 ± 0.9</td>
<td>13.2 ± 1.8</td>
<td>4.7 ± 1.0</td>
<td>1.3 ± 0.6</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>DMDBC 90</td>
<td></td>
<td>3</td>
<td>21.2 ± 2.1</td>
<td>15.0 ± 1.7</td>
<td>6.2 ± 1.6</td>
<td>49.3 ± 1.5</td>
<td>0.17 ± 0.09</td>
</tr>
<tr>
<td>24</td>
<td>Acetone 3</td>
<td>3</td>
<td>9.2 ± 0.5</td>
<td>13.2 ± 2.4</td>
<td>4.0 ± 2.1</td>
<td>3.0 ± 1.0</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>DMDBC 10</td>
<td></td>
<td>3</td>
<td>19.8 ± 1.0</td>
<td>12.2 ± 0.7</td>
<td>7.7 ± 0.5</td>
<td>60.8 ± 2.6</td>
<td>0.14 ± 0.07</td>
</tr>
<tr>
<td>DMDBC 90</td>
<td></td>
<td>3</td>
<td>31.3 ± 1.6</td>
<td>15.5 ± 1.4</td>
<td>15.9 ± 2.8</td>
<td>57.3 ± 8.0</td>
<td>0.14 ± 0.05</td>
</tr>
<tr>
<td>Acetone 3</td>
<td>DMDBC 10</td>
<td>3</td>
<td>8.5 ± 1.1</td>
<td>12.1 ± 2.1</td>
<td>3.6 ± 1.0</td>
<td>2.3 ± 1.2</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>DMDBC 90</td>
<td></td>
<td>3</td>
<td>12.4 ± 1.7</td>
<td>11.7 ± 2.1</td>
<td>0.7 ± 1.4</td>
<td>17.0 ± 4.6</td>
<td>0.21 ± 0.07</td>
</tr>
<tr>
<td>144</td>
<td>Acetone 3</td>
<td>3</td>
<td>9.6 ± 0.4</td>
<td>13.5 ± 2.8</td>
<td>4.2 ± 2.3</td>
<td>0.5 ± 0.7</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>DMDBC 90</td>
<td></td>
<td>3</td>
<td>19.3 ± 4.3</td>
<td>19.4 ± 6.7</td>
<td>0.1 ± 2.3</td>
<td>22.3 ± 7.5</td>
<td>6.68 ± 2.00</td>
</tr>
</tbody>
</table>

Group mean values ± SD. nd, not done.
*Significantly different from control, P < 0.001, Student’s t-test.

Table III. Kinetics of Comet assay values in mouse hepatocytes following skin application of DMDBC

<table>
<thead>
<tr>
<th>Sampling time (h)</th>
<th>Treatment (mg/kg)</th>
<th>No. of animals</th>
<th>Tail moment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>DMN 2.5</td>
<td>11</td>
<td>8.8 ± 2.0</td>
</tr>
<tr>
<td>4</td>
<td>Acetone 5</td>
<td>6</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>DMDBC 10</td>
<td></td>
<td>6</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>DMDBC 90</td>
<td></td>
<td>6</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>15</td>
<td>Acetone 5</td>
<td>6</td>
<td>0.7 ± 0.6</td>
</tr>
<tr>
<td>DMDBC 10</td>
<td></td>
<td>6</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>DMDBC 90</td>
<td></td>
<td>6</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>24</td>
<td>Acetone 5</td>
<td>6</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>DMDBC 10</td>
<td></td>
<td>6</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>DMDBC 90</td>
<td></td>
<td>6</td>
<td>2.1 ± 1.0a</td>
</tr>
<tr>
<td>48</td>
<td>Acetone 5</td>
<td>6</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>DMDBC 10</td>
<td></td>
<td>6</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>DMDBC 90</td>
<td></td>
<td>6</td>
<td>1.8 ± 0.3a</td>
</tr>
<tr>
<td>72</td>
<td>Acetone 5</td>
<td>6</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>DMDBC 10</td>
<td></td>
<td>6</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>DMDBC 90</td>
<td></td>
<td>6</td>
<td>1.7 ± 0.9</td>
</tr>
<tr>
<td>Acetone 5</td>
<td>DMDBC 10</td>
<td>6</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>DMDBC 90</td>
<td></td>
<td>6</td>
<td>0.5 ± 0.2</td>
</tr>
</tbody>
</table>

Group mean values of median TM ± SD.
*Significantly different from control, P < 0.01, Mann-Whitney test.

Lyon, France). Hepatocytes were prepared by using a two-step in situ collagenase perfusion method. The liver was initially perfused with HEPES buffer (10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 0.4 mM Na2HPO4, 12H2O, pH 7.65) at 40°C at a flow rate of 10 ml/min for 5 min, and then with 0.025% w/v collagenase A (Boehringer Mannheim, Mannheim, Germany) plus 0.075% w/v CaCl2 (Sigma, St Louis, MO) for 5 min. Hepatocytes were collected from the perfused liver, filtered through a 60 µm sterile mesh and suspended in William’s medium E (WME) (Gibco BRL, Cergy Pontoise, France) supplemented with 1% fetal bovine serum (FBS) (Gibco). The percentage of viable cells, measured using the Trypan blue exclusion test, always exceeded 75% (data not shown). Isolated hepatocytes were either stored at –80°C until DNA isolation for 32P-post-labeling assay or immediately used for the UDS or Comet assay. Each experiment included vehicle-, DMN- and DMDBC-treated mice.

32P-post-labeling analysis
DNA from frozen hepatocytes was isolated by enzymatic digestion of protein and RNA and solvent extraction as described by Gupta (15). Using the nuclease P1 enhancement procedure of the 32P-post-labeling assay (16), DMDBC–DNA adducts were labeled, separated and quantified as described by Pépin-Roussel et al. (17). Data were expressed as total DMDBC-related DNA adducts/10^8 nucleotides.

UDS assay
Approximately 3×10^5 viable hepatocytes were seeded onto 18 mm diameter round coverslips placed in each well of 6-well culture plates in WME medium supplemented with 50 U/ml penicillin (Gibco BRL), 50 µg/ml streptomycin (Gibco BRL), 2 mM t-glutamine (Gibco BRL), 1 U/ml insulin (Novo Nordisk Pharmaceutics, Boulogne, France), 10^{-6} M hydrocortisone (Roussel, Paris, France) and 10% FBS. Non-attached cells were removed after 2 h. Attached hepatocytes were then incubated in serum-free medium containing 0.37 MBq/ml [3H]thymidine (sp. act. 185 MBq/ml; Amersham, Crétial, France) in a humidified atmosphere at 37°C, 5% CO2. After 4 h incubation the hepatocytes were rinsed and incubated for 15–18 h in serum-free WME medium containing 0.25 mM unlabeled thymidine (Sigma). After the cold chase period the cultures were washed with phosphate-buffered saline at 37°C (Gibco), swollen in 1% sodium citrate solution and fixed in ethanol:acetic acid (3:1 v:v). The dried coverslips were mounted on microscope slides, dipped in Kodak NTB2 emulsion (Integrabioscience, Cergy le Haut, France).
diluted 1:1 with deionized water and exposed for 10 days at 4°C. After development, the slides were stained with Harris hematoxylin solution (Merck, Darmstadt, Germany). One hundred randomly selected nuclei were analyzed on duplicate slides. Silver grains were quantified using image analysis systems (Grain v.1.6, York Electronic Research, Huntington, UK and Domino, Perceptives Instruments, Haverhill, UK) coupled to a Leitz microscope (×100 magnification, oil immersion) and a video camera. All the slides were coded and analyzed by the same operator. The net nuclear grain (NNG) value, defined as the number of grains present in the nucleus minus the mean number of grains in three equivalent areas of cytoplasm adjacent to the nucleus, was recorded. Data were expressed as means ± SD obtained from 100 hepatocytes. The percentage of cells undergoing repair, i.e. cells with an NNG exceeding the threshold value of 5, was also determined (18). Hepatocytes undergoing replicative DNA synthesis can be easily distinguished from hepatocytes undergoing DNA repair by the heavily labeled nuclei. The percentage of hepatocytes in S phase was determined by scoring 3000 randomly selected hepatocytes per animal.

Comet assay
As described by Singh et al. (19), freshly isolated hepatocytes were resuspended at 5×10^5 cells/ml in 0.5% low melting point agarose (Sigma). Seventy-five microliters of the suspension was embedded in an agarose sandwich on fully frosted microscope slides. The slides were immersed in freshly prepared cold lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris base, 1% sodium sarcosinate, pH 10, supplemented with 1% Triton X-100 and 10% dimethylsulfoxide) for at least 1 h at 4°C, then transferred to a horizontal electrophoresis tank filled with electrophoresis buffer (1 mM Na2EDTA, 300 mM NaOH, pH 13.5) for 20 min at 4°C in the dark to allow DNA to unwind before electrophoresis for 15 min at 25 V and 300 mA. After electrophoresis the slides were washed in neutralization buffer (0.4 M Tris, pH 7.5) to remove excess alkali and detergent and then stained with 50 µl of aqueous propidium iodide (20 µg/ml). One-hundred and fifty randomly selected cells were analyzed on duplicate slides using an image analysis system (Komet v.3.0; Kinetic Imaging Ltd, Liverpool, UK) coupled to a Zeiss fluorescence microscope (×250 magnification) and a CDD camera. All the slides were processed simultaneously, coded and analyzed by the same operator. The tail moment (TM), defined as the product of the relative amount of DNA in the comet tail and the length of the comet measured from the trailing edge of the cell, was recorded. Data were expressed as the median of the 150 TM values obtained per animal. Additional parameters, such as the percentage of tail DNA and tail length were also recorded, and were in keeping with the TM values (data not shown).

Statistical analysis
Student’s t-test was used to analyze the UDS response and the percentage of S phase hepatocytes. The Mann–Whitney test was used to analyze the Comet assay data.

Results
Tables I–III summarize the number of DNA adducts and the NNG and TM values, respectively, from 4 to 144 h after treatment with 10 or 90 mg/kg DMDBC. Figure 1 illustrates the kinetics of the three parameters.

32P-post-labeling analysis
As previously described by Pépin-Roussel et al. (5), the chromatograms of DMDBC-related DNA adducts after 32P-post-labeling showed a complex pattern of at least 17 different spots that were reproducibly observed in the liver of mice treated with both doses and at all sampling times. The control map bore no detectable DMDBC-related DNA adducts after an exposure time 3-fold longer than for the DMDBC maps (data not shown).

A continuous increase in the amount of DNA adducts was observed from 4 to 24 h after skin application of 10 mg/kg (Table I). At 24 h post-treatment, a steady-state level of 423 adducts/10^8 nucleotides was reached and persisted over the entire observation period. In the liver of mice treated with 90 mg/kg, the number of DNA adducts increased sharply from 4 to 24 h, reaching a maximum of 8814 adducts/10^8 nucleotides at 24 h. The number of DNA adducts then fell strongly at 48 and 72 h. After a post-application period of 144 h, 481 adducts/10^8 nucleotides, representing 5% of the peak value, still persisted.

UDS assay
The NNG of hepatocytes from control animals ranged from −4.7 to −3.6 and the proportion of cells undergoing DNA repair ranged from 0.10 to 0.15% during the study. These results are consistent with previous data (20).

DMN, used as a positive control, significantly increased UDS activity. Two hours after oral administration of 2.5 or 10 mg/kg, NNG counts reached +29.6 and +76.3, respectively (P < 0.001). The proportion of hepatocytes undergoing excision repair was 77.8 and 95.7%, respectively. These results are in good agreement with published data (18).

Following skin application of 10 mg/kg, a clear UDS response was observed at the 15 h sampling time (+6.2 NNG, P < 0.001) (Table II). The induction of UDS was maximal 24 h after treatment. The NNG count reached +7.7 (P < 0.001) and 60.8% of hepatocytes were undergoing repair. Seventy-two hours after exposure the NNG count declined to +0.7, a value no longer statistically different from the control. However, 17.0% of hepatocytes were still undergoing repair. Following exposure to 90 mg/kg DMDBC, UDS peaked as
early as 15 h; the NGG count reached +24.5 (P < 0.001) and 64% of cells were undergoing repair. NNG counts then fell steadily to +15.9 and +6.6 at 24 and 72 h (P < 0.001), respectively, returning to a value no longer statistically different from the control at 144 h (~0.1 NNG). However, 22.3% of hepatocytes were still undergoing repair at 144 h.

The percentage of control hepatocytes undergoing replicative DNA synthesis ranged from 0.10 to 0.15% from 4 to 144 h, values consistent with published data (21). After skin application of 10 mg/kg DMDBC, no changes in the percentage of S phase cells were observed at any time. Following exposure to 90 mg/kg DMDBC, no changes were observed at 15 and 24 h, while 14.6 and 6.7% of hepatocytes were undergoing replicative DNA synthesis at 72 and 144 h, respectively.

**Comet assay**

The TM in the Comet assay of control hepatocytes ranged from 0.5 to 0.9 between 4 and 144 h (Table III). When level of DNA adducts increased sharply to a maximum at 24 h, while 14.6 and 6.7% of hepatocytes were undergoing repair, NER values consistent with published data (21). After skin application of 90 mg/kg DMDBC, no changes in the percentage of S phase cells were observed at any time. Following exposure to 90 mg/kg DMDBC, no changes were observed at 15 and 24 h, while 14.6 and 6.7% of hepatocytes were undergoing replicative DNA synthesis at 72 and 144 h, respectively.

**Discussion**

Given the marked differences in the magnitude of MF 28 days after in vivo exposure of the Mutat™Mouse to 10 or 90 mg/kg DMDBC, we investigated whether similar differences could be observed in the early stages of the genotoxicity process. In the present study, the induction and removal of DNA adducts were monitored in mouse hepatocytes from 4 to 144 h after a single skin application of 10 or 90 mg/kg DMDBC.

On exposure to 10 mg/kg DMDBC, DNA adduct levels reached a plateau as early as 24 h and then remained unchanged until the last sampling time of 144 h (Figure 1). UDS activity was rapidly induced, reaching a maximum at 24 h and then falling to a near baseline level at 72 h. This time course of DNA repair induction, with an early peak and a decline to baseline after 24–48 h, is consistent with that described for other liver carcinogens, like 2-acetylamino fluorene and 7,12-dimethylbenz[a]anthracene, and more recently.

The slight but significant increases in TM observed in the Comet assay 24 and 48 h after exposure to 90 mg/kg DMDBC could reflect the accumulation of incomplete repair intermediates in response to the impairment of DNA repair mechanisms. Similar results have been produced in vitro using DNA repair inhibitors to reveal or amplify the effects of several polycyclic aromatic hydrocarbons when tested with the Comet assay (30–32). The slight increases in TM could also reflect DNA strand breaks occurring secondarily through the formation of depurinating DNA adducts and resulting alkali-labile abasic sites, as already described for benzo[a] pyrene and 7,12-dimethylbenz[a]anthracene, and more recently.
We are grateful to Ms Marie-Jose Plessis (Institut Curie) for her excellent assay in the liver of rats exposed to 2-acetylamino-7H-Dibenzo[c,g]carbazole. This work received financial support from the European Environmental Program.

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References


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