CLH report

Annex I

International Chemical Identification:

1,4-Benzenediamine, N,N'-mixed Ph and tolyl derivs. ;

Reaction mass of N-phenyl,N’-o-tolyl-phenylene diamine, N,N’-diphenyl-p-phenylene diamine and N,N’-di-o-tolyl-phenylene diamine

EC Number: **273-227-8**

CAS Number: **68953-84-4**

Index Number: **-**

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1 HUMAN HEALTH HAZARD ASSESSMENT

1.1 Reproductive toxicity studies with BENPAT

1.1.1 Two-Generation Reproductive Toxicity Study (OECD TG 416)

Reproductive Toxicity of BENAPT was investigated in a GLP-compliant two-generation reproductive toxicity study, performed according to OECD TG 416 (RTI, 2001a). Rats (Sprague-Dawley) received dietary doses of BENPAT at 0, 120, 400, and 1500 ppm (corresponding to approx. 0, 7.5, 25, and 100 mg/kg bw/d of BENPAT, vehicle: corn oil). The DS considers this study as a key study for adverse effects on sexual function and fertility, and on development. Test animals were exposed as follows: F0 male & females - continuously for 10 weeks prior to mating, and then during the mating period. F0 females & males continued on diet until deliveries occurred. Female exposures continued through a 21-day lactation period post-delivery. For F1 generation rats, animals were exposed via diet upon their ingestion of maternal diet. Their exposures to both sexes continued through a 10-week period after which housing was arranged for mating of F1 animals. Again, exposures continued for females through the gestation and lactation periods post-deliveries whereas F1 male exposures ended upon F2 deliveries. The F2 generation was not exposed to treated diets except late in the 21-day lactation period (diets, when offered, are available 24 hr/day, 7 days/week).

1.1.1.1 Characterisation of polycystic kidneys

"Polycystic kidneys were characterized by the presence of renal tubule cysts primarily in the outer medulla but occasionally in the inner medulla (papilla) and cortex. In general, cysts were lined by cuboidal to flattened epithelial cells and were surrounded by a variable amount of fibrous tissue and chronic-active inflammation. The majority of the cysts appeared empty; however, some contained either red blood cells, mixed inflammatory cells, desquamated tubular epithelial cells or a mixture of the aforementioned cells…", cited from the Two-Generation Reproductive Toxicity Study with BENPAT, OECD TG 416 (RTI, 2001b).

1.1.1.2 Body and organ weights of parental animals and weanlings, cited from Two-Generation Reproductive Toxicity Study with BENPAT (OECD TG 416)

F0 male body weight "exhibited a statistically significant reduction at 1500 ppm [100 mg/kg bw/d] for all weeks evaluated, through the end of the pre-breed dosing period (day 70) and through the two-week mating period (to day 84) […]. F0 male weekly body weight changes exhibited statistically significant reductions at 1500 ppm [highest dose] […] for the entire pre-breed period (sd 0-70), at 400 ppm [mid dose] and 120 ppm [low dose] for week 3 (sd 14-21), and at 120 ppm [low dose] for week 8 (sd 49-56)." During pre-breeding and mating, "F0 male feed consumption, expressed asg/day, was significantly lower at 1500 ppm [100 mg/kg bw/d] only for week 1 (days 0-7) during the ten-week pre-breed dosing period. When the data were expressed as g/kg/day, the values at 1500 ppm [100 mg/kg bw/d] were significantly increased for week 5 (sd 28-35), 7 (sd 42-49), 9 (sd 56-63) and 10 (sd 63-70)… "

F0 female body weight of high dose animals were significantly lower, "beginning at the end of week 1 (pre-breed sd day 7) and continuing through nine of the ten-week pre-breed exposure period. […] F0 female weekly body weight changes [during pre-breeding] exhibited only a statistically significant reduction, at 1500 ppm [100 mg/kg bw/d] for week 1 (sd 0-7), and only a statistically significant increase, at 400 ppm [25 mg/kg bw/d] for week 10 (sd 63-70)." F0 female feed consumption (g/day) was significantly increased at mid dose for week 10 (sd 63-70) during the ten-week pre-breed dosing period. "When the data were expressed on g/kg/day, maternal feed consumption values were significantly increased at 400 ppm [25 mg/kg bw/d] for week 1 (sd 0-7), 5 (sd 28-35), 6 (sd 35-42), and for the entire pre-breed period (sd 0-70), and at 400 [mid] and 1500 ppm [high dose] for weeks 7 (sd 42-49), 8 (SD 49-56) and 10 (SD 63-70)." F0 female body weight and body weight gain during gestation were statistically significantly reduced at 100 mg/kg bw/d. Maternal gestational feed consumption (g/day) was significantly reduced at high dose for GD 7-14; "when expressed as g/kg/day, feed consumption was significantly increased at 400 [mid] and 1500 ppm [high dose] for gd 14-20. […]"

Body weight of dams during lactation was statistically significantly reduced at 100 mg/kg bw/d for PND 0, 4, and 7, but lactation weight change (PND 0-21) was significantly increased at the highest dose.

F0 male body weight at scheduled sacrifice was significantly reduced at 100 mg/kg bw/d. In F0 male animals," relative liver weights were significantly increased (109.2 % of control value) at 1500 ppm [100 mg/kg bw/d]. Relative paired kidney weights were significantly increased at 400 ppm [25 mg/kg bw/d] (106.0 % of control value) and at 1500 ppm [100 mg/kg bw/d] (110.7 % of control value)"; there were no apparent treatment-related gross necropsy findings in males. There were no apparent treatment- or dose-related findings in males who died on study or who were sacrificed on schedule. Two kidneys at 120 ppm [low dose] exhibited "pits" and one kidney at 1500 ppm [100 mg/kg bw/d] exhibited a clear cyst on the surface. Similarly, there were no treatment- or dose-related histopathologic findings in F0 male reproductive organs or in organs with gross lesions (including retained kidneys)."[…]

F0 female terminal "body weights were statistically equivalent across groups (with a significant downward trend, $p<0.05$)." […] In F0 females, "relative liver weights were significantly increased at 1500 ppm [100 mg/kg] bw/d (119.8 %)[at scheduled sacrifice]. Relative paired kidney weights were significantly increased at 400 ppm [25 mg/kg bw/d] (108.7 % of control) and 1500 ppm [100 mg/kg bw/d] (112.0 % of control). [Furthermore,] relative paired ovary weight was statistically significantly reduced at 120 ppm [7.5 mg/kg bw/d] (88.0 % of control) and increased, but not statistically significant, at 400 ppm [25 mg/kg bw/d] (130.9 % of control) and at 1500 ppm [100 mg/kg bw/d] (168.0 % of control). Relative uterine weights were significantly reduced at 400 ppm [25 mg/kg bw/d] (79.3 %) and at 1500 ppm [100 mg/kg bw/d] (80.4 %). […] At scheduled sacrifice of surviving females, gross effects (probably treatment-related) were observed on the kidneys at 120 ppm [7.5 mg/kg bw/d] (one animal) and at 1500 ppm [100 mg/kg bw/d] (three females)." […] Histopathologic findings in F0 females were observed at 100 mg/kg bw/d in kidneys ("specifically polycystic and cortical necrosis") and livers ("specifically hematopoietic cell proliferation, hepatocellular centrilobular necrosis").

"**F1 weanling [PND 21] male body weight** was significantly increased (109.2 % of control) at 400 ppm [25 mg/kg bw/d] (and statistically but not significantly reduced at 1500 ppm [100 mg/kg bw/d], 96.4 % of the control value). […] Relative male thymus weight (as percentage of sacrifice weight) was significantly reduced at 1500 ppm [100 mg/kg bw/d] (91.7 % of controls); relative male spleen weight was significantly increased at 120 ppm [7.5 mg/kg bw/d] (113.7 % of control) and 400 ppm [25 mg/kg bw/d] (123.8 % of control) and slightly, but not statistically significantly, increased (113.2 % of control) at 1500 ppm [100 mg/kg bw/d]."

F1 male body weights of high dose animals were significantly reduced during the last three weeks of the pre-breeding period and during mating. F1 male body weight changes were significantly reduced at 100 mg/kg bw/d and 25 mg/kg bw/d "for the entire 70-day pre-breed period. […] Feed consumption [(g/day)] was significantly reduced at 400 ppm [mid dose] and 1500 ppm [high dose] for weeks 7 (sd 42-49) and 10 (sd 63-70). When feed consumption data were expressed as g/kg/day, feed consumption was significantly decreased at 120 ppm [low dose] for week 1 (sd 0-7) and at 400 ppm [mid dose] for week 8 (sd 49-56) during the 12-week period. […]

F1 weanling (PND 21) female body weight "was significantly increased at 400 ppm [25 mg/kg bw/d] (109.1 % of control value)." […] Relative F1 female spleen weight on PND 21 "was significantly increased at 120 ppm [7.5 mg/kg bw/d] (112.7 % of controls), [25 mg/kg bw/d] 400 ppm (113.8 % of controls) and at 1500 ppm [100 mg/kg bw/d] (116.6 %). Relative female brain weight was significantly reduced at 400 ppm [25 mg/kg bw/d] (88.8 % of control) and at 1500 ppm [100 mg/kg bw/d] (89.8 % of control)."

F1 female body weights and weight gains during the ten-week pre-breed dosing period […] were essentially unaffected." […] During gestation, "maternal gestational body weights were statistically equivalent for all time points." However, "maternal gestational weight change (gd 0-20) was significantly reduced at 400 [25 mg/kg bw/d] and 1500 ppm" [100 mg/kg bw/d]. Food consumption of dams, expressed as g/kg/day, was significantly increased at high dose for gd 14-20. During lactation maternal F1 body weight "exhibited significant reductions at 1500 ppm on PND 0, 4, and 7."

At scheduled sacrifice, F1 male body weight was significantly reduced at 100 mg/kg bw/d. […] F1 male relative liver weights and relative paired kidney weights were significantly increased at 100 mg/kg bw/d (108.8 % of control liver weight and 110.1 % of control kidney weight). Gross findings in F1 males "were limited to the kidneys" (hydronephrosis, polycystic kidneys, irregular renal cortex, the latter two parameters being dose-dependent)

At scheduled sacrifice, F1 female body weight "was equivalent across all groups". Furthermore, relative liver weight was increased at 100 mg/kg bw/d (112.2 %), but not statistically significantly different from control. Relative uterine weights of the mid and high dose groups were decreased, but not statistically significantly (80.7 % and 81.2 % of the control value, respectively). Additionally, "relative paired ovary weight was significantly reduced at 400 ppm [25 mg/kg bw/d] (82.4 % of controls); the value at 1500 ppm [100 mg/kg bw/d] was 91.2 % of controls, not statistically significantly different." […] Gross findings in F1 females were observed in the kidneys at 100 mg/kg bw/d. Microscopic treatment-related findings were limited to the kidneys in all dose groups (dose-dependent; tubule dilatation in the renal papilla, chronic inflammation, nephropathy, polycystic kidneys, and renal tubule regeneration).

Body weights of male F2 weanlings (PND 21) "were significantly increased at 120 ppm [7.5 mg/kg bw/d] and 400 ppm" [25 mg/kg bw/d], compared to controls.[…] "Male relative brain weight was significantly reduced at 1500 ppm [7.5 mg/kg bw/d] (93.8 % of controls) and 400 ppm [25 mg/kg bw/d] (87 % of controls)". […] F2 weanling female body weight was significantly increased at 400 ppm [25 mg/kg bw/d.] […] Relative female brain weight was significantly reduced at 400 ppm [25 mg/kg bw/d] (91.4 % of controls). […] All of the gross findings involved the kidneys (except for one male at 1500 ppm [100 mg/kg bw/d] with an enlarged eye and polycystic kidneys)." Examinations revealed "renal lesions presented as one or more clear cysts on the surface or within the kidney, one or more white foci on the surface, and hydronephrosis (dilation of renal pelvis), predominantly at 1500 ppm [100 mg/kg bw/d]. […] Microscopic findings were also limited to the kidneys.; treatment-related lesions included polycystic kidneys" in males and females at 7.5, 25, and 100 mg/kg bw/d (RTI, 2001a).

1.1.2 One-generation mechanistic study - study design

The objective of this study was to provide additional information on the previous findings from a multi-generation reproductive toxicity study and a chronic study of dietary Wingstay 100 (BENPAT) in rats. The study design involved 5 groups: group 1 is a control group and groups 2-5 are dosed with 2500 ppm of Wingstay 100 via the diet. The dose groups were differentiated by the exposure duration and timing. Group 2 was dosed only during pre-breed and mating. Group 3 was dosed only during gestation and lactation. Group 4 was dosed during all phases: pre-breed, mating, gestation and lactation. Group 5 was also dosed during all phases, but additionally received 600 ppm iron supplementation via the drinking water. Duration of the phases were as follows: 4 weeks of pre-breeding; up to 2 weeks of mating phase (depending on when the females became sperm positive); 3 weeks of gestation phase; 3 weeks of lactation phase. Males and females were paired within groups (1:1) for the 2-week mating period. Once a given female was found sperm positive (= date designated GD 0) "her" male was euthanized and discarded. Other than mating periods, animals were housed individually. On [PND] 4, litters were culled to 10, counted, sexed and weighed. On [PND] 7, 14 and 21, pups were counted, sexed and weighed. All F1 pups were euthanized and one/sex/litter necropsied on [PND] 21. [F0] Females were euthanized on [PND] 21 and maternal blood samples were assessed: WBC, RBC, Platelet count, Hgb, Hct, MCV, MCH, MPV, MCHC, RDW, WBC differential and methemoglobin. Male animals were not examined histopathologically. The following maternal organs were weighed and retained in fixative: spleen, liver, kidneys (2). Kidneys of the control group and of Group 5 were examined histopathologically. The following organs from the one pup/sex/litter, for a maximum of ten/sex/group (and 80 total), were weighed and retained in fixative: spleen, liver, kidneys (2) and heart. The retained offspring kidneys from all groups were examined histologically. Dead pups [on PND] 0 and 1 were examined macroscopically for polycystic kidneys. All dead pups during lactation were necropsied, if possible, for cause of death.

1.2 Reproductive/Developmental toxicity studies with BENPAT constituent DPPD

Table 1: Summary table of other studies relevant for toxicity on sexual function and fertility – studies with BENPAT constituent DPPD

<table>
<tr><th>Type of study/data</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th></tr>
<tr>
<td>Reproductive/ Developmental Toxicity Screening Test, according to OECD TG 421

Reliability: 2, reliable with restriction

(no study report available to the DS)

None GLP-compliant

<u>Deviations:</u>

Dams with offspring killed on day five post-partum, instead of on day 13

(Matsumoto et al., 2013)</td>
<td>Dosed by gastric intubation of N,N’-diphenyl-p-phenylene-diamine (DPPD) at 0 (control: sodium carboxymethyl cellulose), 8, 50, or 300 mg/kg bw/d

CAS: 74-31-7

Purity: 99.9 % (Lot No. KWR0015, purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan))</td>
<td>Rats, [Crl:CD(SD)] SPF, N=13/sex/group

Exposure males: 42 days in total, starting with a 14 days prebreed period;

Exposure females: 42-46 days in total, starting with a 14 days prebreed period, dosing continued throughout mating and gestation period, until four days of lactation</td>
<td><u>F0 generation - effects on sexual function and fertility</u>
<u>300 mg/kg bw/d</u>
↑ Gestation length
<u>50 mg/kg bw/d</u>
↑ Gestation length

Dose-dependent ↓ no. of pups born, delivery index, no. of live pups, birth index, and live birth index on PND 0 (not statistically significant)

<u>F0 generation - general toxicity</u>
No effects on BW, BWC, and feed consumption
<u>300 mg/kg bw/d</u>
Two ♀ died/ sacrificed GD 23 due to dystocia; hemorrhage in uterus lumen, incomplete retention, red colour in lungs, and dark red medulla and hardness on kidneys; for another female nesting and nursing were not observed, and this female was sacrificed on day 1 of lactation due to total litter loss.

One ♀ (50 mg/kg bw/day) sacrificed on day 9 of administration for incorrect operation at time of dosage

<u>F1 generation - general toxicity</u>
<u>300 mg/kg bw/d</u>
↓ live pups and viability index on PND 4 (not statistically significant)

<table>
<tr><th>DPPD [mg/kg bw/d]</th><th>0</th><th>8</th><th>50</th><th>300</th></tr>
<tr><td>Fertility index [%]</td><td>100</td><td>92.3</td><td>100</td><td>100</td></tr>
<tr><td>No. with live litters, PND 0</td><td>13</td><td>12</td><td>12</td><td>11</td></tr>
<tr><td>No. of dams died/sacrificed during gestation and lactation</td><td>0</td><td>0</td><td>0</td><td>3</td></tr>
<tr><td>Gestational length [d]</td><td>22.4 ± 0.5</td><td>22.8 ± 0.5</td><td>23.0 ±0.0[1]</td><td>23.0 ± 0.4[1]</td></tr>
<tr><td>No. implantations</td><td>15.9 ± 1.5</td><td>16.3 ± 2.7</td><td>16.2 ± 1.0</td><td>15.8 ± 1.9</td></tr>
<tr><td>No. of pups born, PND 0</td><td>14.8 ± 2.1</td><td>14.8 ± 3.1</td><td>14.3 ± 1.5</td><td>13.7 ± 3.1</td></tr>
<tr><td>No. live pups/litter, PND 0</td><td>14.7 ± 2.1</td><td>14.4 ± 2.7</td><td>13.8 ± 1.5</td><td>12.8 ± 4.1</td></tr>
</table>
</td>
</tr>
</table>

[1] Significantly different from the control group, $p < 0.01$, Kruskal–Wallis followed by the Dunnett type test (Matsumoto et al., 2013).

<table>
<tr><th>Type of study/data</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th></tr>
<tr><td></td><td></td><td></td><td>
<table>
<tr><td>Live birth index [%]</td><td>99.5 ± 1.7</td><td>97.7 ± 5.4</td><td>97.2 ± 5.3</td><td>92.0 ± 20.7</td></tr>
<tr><td>Sex ratio [% males]</td><td>44.3 ± 18.3</td><td>39.4 ± 12.1</td><td>47.6 ± 14.1</td><td>48.1 ± 13.2</td></tr>
</table>
</td></tr>
<tr><td>Non-guideline study,

Reproductive studies on DPPD and its impurities as a food additive to the diets of white rats

Reliability: 4, not assignable

(Study report not available to DS, data available from US EPA provisional peer-reviewed toxicity values for DPPD)

GLP-none compliant

(EPA-US, 2009)</td><td><u>DPPD</u>, diets of 300 and 1000 ppm, corresponding to approx. 31 and 103 mg/kg/bw day (EPA-US, 2009)

CAS: 74-31-7

Purity: 99.5 %</td><td>Rats, Wistar; N=approx. 20/ group

F0 ♀ exposed during pregnancy and lactation

F0 ♂ exposed to control diet, except for pairing with females (same diet females were exposed to)

Analysis of minimum, maximum and mid-point of estimated gestation, number of live and dead offspring, number of pups raised to 21 days, and weight of pups on PND 21; No data on maternal food consumption or body weight</td><td>- ↑ no. of dams that hemorrhaged abnormally during delivery at all doses of DPPD
- ↑ no. of anemic dams (among survivors) at all doses of DPPD

Data for F1 effects on sexual function and fertility of F0 generation and for offspring toxicity are summarised below:

<table>
<tr><th>DPPD [mg/kg bw]</th><th>Fertility rate [%]</th><th>Maternal mortality [%]</th><th>Mean live births[2]</th><th>Mean dead births[2]</th><th>Mean gestation time [d][3]</th><th>Litters weaned [%][4]</th><th>Mean weanling weight, PND 21 [g]</th></tr>
<tr><td>0 (Control)</td><td>85</td><td>0</td><td>9.0</td><td>0.4</td><td>20.9</td><td>80</td><td>46.1</td></tr>
<tr><td>31</td><td>75</td><td>5 (1/20)</td><td>4.6</td><td>4.1</td><td>22.6</td><td>38</td><td>47.0</td></tr>
<tr><td>103</td><td>81</td><td>10 (2/20)</td><td>1.9</td><td>5.4</td><td>22.6</td><td>7.7</td><td>36.0</td></tr>
</table>
Table adopted from (EPA-US, 2009)</td></tr>
<tr><td>Non-guideline study,

Antioxidants, prostaglandins and preterm labor

Reliability: 2, reliable with restriction</td><td>NN’-Diphenyl-p-phenylene-1-4-diamine (DPPD), in olive oil

Purity: No data</td><td>Adult pregnant Wistar rats were subcutaneously dosed with DPPD at:

200 mg/kg bw/d from GD14 (N=24) and GD17(N=8) until parturition,

300 mg/kg bw/d (N=16) and</td><td>- 200 mg/kg bw/d DPPD from GD 17: upsets parturition

- 400 mg/kg bw/d DPPD from GD14: prolongs pregnancy duration, often prevents birth of living fetuses; causes female mortality during delivery

- Increased number of non-delivering females (DPPD-treated) with living fetus in utero at day 25 of pregnancy:</td></tr>
</table>

[2] “Ashe (1956) reports that these values may not be absolutely accurate due to unquantifiable cannibalism, but they are still useful for comparing across groups” (EPA-US, 2009).

[3] Data are uncertain, timing of conception was not accurately determined.

[4] “Number litters weaned divided by the number dams pregnant × 100; determined on Postnatal Day 21” (EPA-US, 2009).

<table>
<tr><th>Type of study/data</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th></tr>
<tr>
<td>GLP: No information

(Marois, 1998)</td>
<td></td>
<td>400 mg/kg bw/d (N=12) from GD14until parturition,

N=10/control group

♀ treated with 400 mg/kg bw DPPD were subcutaneously injected with 500 µg/kg bw Prostaglandin F2α (PGF2α), four times, every hour from GD20 or 21

Examination: gestation length; non-delivering ♀ were sacrificed at 25d or 26d of pregnancy; analysis of no. & viability of pups</td>
<td>
<table>
<tr><th>DPPD [mg/kg bw/d]</th><th>Number of ♀, sacrificed day 25 of pregnancy</th><th>Number of living fetus in utero</th></tr>
<tr><td>200</td><td>4/24</td><td>11 (per ♀)</td></tr>
<tr><td>300</td><td>6/16</td><td>25 (in total)</td></tr>
<tr><td>400</td><td>8/12</td><td>10 (in total)</td></tr>
</table>
- 2 mg/kg bw (in total) of PGF2α injected on GD21 almost totally cancel effects of DPPD
<table>
<tr><td>No. ♀</td><td>10</td><td>6</td><td>4</td><td>12</td><td>6</td><td>6</td></tr>
<tr><td>PGF2α injection, [d] of gestation</td><td>-</td><td>20</td><td>21</td><td>-</td><td>21</td><td>22</td></tr>
<tr><td>DPPD injection, [d] of gestation</td><td>-</td><td>-</td><td>-</td><td>14</td><td>14</td><td>14</td></tr>
<tr><td>Date of parturition, [d] of gestation</td><td>22-23</td><td>21 - 22</td><td>22</td><td>23-25*</td><td>21 -22</td><td>22 - 23</td></tr>
<tr><td>Average number of living fetus/ ♀</td><td>10-15</td><td>3.7</td><td>9</td><td>1.3</td><td>6</td><td>8.3</td></tr>
</table>
PGF2a: 2 mg/kg bw in total
DPPD: 400 mg/kg bw/d
* Eight ♀ sacrificed on day 25
</td>
</tr>
<tr>
<td>Non-guideline study,

Inhibition of prostaglandin synthesis in rabbit kidney medulla slices by antioxidants

Reliability: 2, reliable with restriction

GLP: No information

(Fujimoto et al., 1984)</td>
<td>NN'-Diphenyl-p-phenylene-1-4-diamine (DPPD)</td>
<td>Kidney medullary slices were prepared from ♀ rabbits and incubate with antioxidants (DPPD, 1 µM); medium was assayed for prostaglandin E (PGE), using HPLC (PGE extracted with ethyl acetate (approx. pH3) was measured after based-catalysed conversion to prostaglandin B);

Slices were incubated in presence of arachidonic acid, to stimulate prostaglandin synthesis</td>
<td>- Treatment with DPPD (1 µM) resulted in decrease of PGE release from rabbit kidney medulla slices

- Dose-dependent stimulation of PGE production after treatment of slices with arachidonic acid

- Arachidonic acid (50, 100 µM) induced stimulation of PGE formation was not blocked by DPPD

- The authors suggest that DPPD inhibits prostaglandin formation by affecting a phospholipase pathway (not affecting the cyclooxygenase enzyme)</td>
</tr>
<tr>
<td>Non-guideline study,

Effect of DPPD on the Reproductive Process in the Rat</td>
<td>Dietary doses of <u>DPPD</u>, at 0.0125, 0.0625, 0.313, and 1.55 %, corresponding</td>
<td>Rats (strain: No information), N=10-17 ♀

Exposure: starting 10 days prior to mating, during</td>
<td>- Signs of difficult parturition occasionally observed (vaginal bleeding, prolapse of the uterus) at all doses of DPPD

- ↑ pup mortality at all doses of DPPD (deaths occurred mainly during parturition)

- Maternal mortality at all doses of DPPD (deaths occurred mainly during parturition)</td>
</tr>
</table>

<table>
<tr><th>Type of study/data</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th></tr>
<tr><td>Reliability: 4, not assignable
Only abstract available (data available from US EPA provisional peer-reviewed toxicity values for DPPD)
GLP-none compliant
(Ames et al., 1956)</td><td>to approx. 11, 55, 275, and 1360 mg/kg bw/ day (EPA-US, 2009)
CAS: 74-31-7
Purity: No data “feed grade”</td><td>parturition and lactation
Examinations: Fertility index (no. pregnant ♀/ no. mated ♀), litter efficiency (% pregnant ♀ with ≥1 viable foetus), mortality index (no. dams dying at parturition/no. pregnant ♀), duration of pregnancy, litter size, viability index (no. pups alive PND 3/ no. pups born), and lactation index (no. young weaned/ no. alive PND 3)</td><td>
<table>
<tr><th>DPPD [mg/kg bw/day]</th><th>Mean duration of gestation [d]</th><th>Mean litter size</th><th>Maternal mortality</th><th>Pup mortality (PND 3)</th></tr>
<tr><td>0</td><td>23</td><td>10.6</td><td>1/17</td><td>33/104 (32 %)</td></tr>
<tr><td>11</td><td>24</td><td>7.9</td><td>0/12</td><td>75/79 (95 %)[5]</td></tr>
<tr><td>55</td><td>25</td><td>4.9</td><td>5/17</td><td>49/49 (100 %)[5]</td></tr>
<tr><td>275</td><td>25</td><td>5.3</td><td>5/10[5]</td><td>16/16 (100 %)[5]</td></tr>
<tr><td>1360</td><td>25</td><td>4.7</td><td>7/13[5]</td><td>14/14 (100 %)[5]</td></tr>
</table>
Table adopted from (EPA-US, 2009)</td></tr>
<tr><td>Non-guideline study,
Investigations of DPPD on gestation and parturition in rats
Reliability: 2, reliable with restriction
GLP-none compliant
(Oser and Oser, 1956)</td><td>Dietary doses of N,N'diphenyl-p-phenylenediamine (<u>DPPD)</u> at 0.025, 0.10, 0.40, and 1.60 %, corresponding to approx. 22, 88, 350, and 1400 mg/kg bw/ day (EPA-US, 2009)
Purity: ≥95 %
(Good-rite DPPD feed grade, a product of the B. F. Goodrich Co.)</td><td>Rats (strain: No information), N=10 ♀/dose (each having previously produced and weaned a normal litter)
Exposure F0 generation: Prebreed (two weeks), mating, parturition, and lactation
Examinations for body weight, duration of pregnancy, number and weight of pups cast or found in utero, mortality up to the end of a normal gestation and lactation period</td><td>- ↓ post-partum survival of dams at all doses of DPPD
- no evidence of organic injury of the pituitary, after histopathological examinations of rats that died in parturition (GD 24 and 25, N=5), compared to normal rats (sacrified GD 22),
- ↑ body weight (>10 %) of pups born dead or found in uterus of ♀ who died at parturition (pups born alive were of normal weight), at all doses of DPPD
- normal proportion of pregnancies at all doses
<table>
<tr><th>DPPD [mg/kg bw/day]</th><th>Duration of gestation mean ±SEM [d]</th><th>Litters born complete (partial)</th><th>Maternal mortality, parturition</th><th>Pups mortality (PND 0)</th><th>Mean pup weight, born dead or in utero (born alive) [g]</th></tr>
<tr><td>0</td><td>22.1± 0.23</td><td>10</td><td>0/10</td><td>18/107 (17 %)</td><td>5.5 (5.5)</td></tr>
<tr><td>22</td><td>22.9± 0.23[6]</td><td>9</td><td>1/10</td><td>42/79 (53 %)[7]</td><td>6.6 (6.2)</td></tr>
<tr><td>88</td><td>24.1± 0.30[6]</td><td>7</td><td>3/10</td><td>21/35 (60 %)[7]</td><td>6.3 (5.4)</td></tr>
<tr><td>350</td><td>25.2± 0.68[6]</td><td>6 (1)</td><td>3/10</td><td>18/20 (90 %)[7]</td><td>6.6 (5.0)</td></tr>
<tr><td>1400</td><td>24.7± 0.54[6]</td><td>4 (3)</td><td>5/10[7]</td><td>20/24 (83 %)[7]</td><td>5.9 (5.0)</td></tr>
</table></td></tr>
</table>

5 Statistically significantly different from control ($p < 0.05$) by Fisher exact test conducted for the “Provisional peer-reviewed toxicity values for DPPD” (EPA-US, 2009).

6 “Statistically significant difference from controls, Student’s t-test, p-value not specified” (EPA-US, 2009)

7 Statistically significant difference from controls, ($p < 0.05$) by Fisher exact test conducted for the “Provisional peer-reviewed toxicity values for DPPD” (EPA-US, 2009)

Numerous animal studies are available, that analysed the effect of BENPAT constituent N,N'-Diphenyl-p-phenylenediamine (DPPD) on developmental toxicity, especially on gestation and parturition. Because studies show similar effects comparable to those of BENPAT, the DS decided to include studies performed with DPPD as supporting evidence. Furthermore, studies with DPPD hypothesise a possible mode of action of BENPAT.

In a reproductive/ developmental toxicity screening test, according to OECD TG 421, Matsumoto and colleagues (Matsumoto et al., 2013) gastric intubated rats with DPPD at doses of 0, 8, 50, or 300 mg/kg bw (Matsumoto et al., 2013).

Furthermore, numerous none-guideline studies from the 1950s were found during literature, addressing an effect of DPPD on gestation and parturition in rats. Ashe (1965) fed Wistar rats with diets of 300 and 1000 ppm (corresponding to 31 and 103 mg/kg bw/d of pure DPPD (EPA-US, 2009)). Study report was not available to the DS. However, data were adopted from US EPA's "Provisional Peer-Reviewed Toxicology Values for DPPD" (EPA-US, 2009). US EPA reported that some data of this study are uncertain, due to e.g. inaccurate determination of timing of conception. Furthermore, a statistical analysis is missing.

US EPA reviewed another non-guideline study, not available by the DS. In this study female rats of unspecified strain were exposed with dietary doses of DPPD, at concentration of 0.0125, 0.0625, 0.313, and 1.55 % (corresponding to an average intake of approx. 11, 55, 275, and 1360 mg/kg bw/day (Ames et al., 1956; EPA-US, 2009)). Ames and colleagues investigated females during pre-breed, parturition and lactation. Examinations were performed for the fertility index, litter efficiency, and mortality index. Furthermore, duration of pregnancy, litter size, a viability index, and a lactation index were analysed.

Finally, (Oser and Oser, 1956) investigated the effect of DPPD in female rats (unspecified strain) from a breeding colony; each one of them has previously produced and weaned a normal litter. Females were fed diets with DPPD concentrations of 0.025, 0.10, 0.40, and 1.60 % (corresponding to approx. 22, 88, 350, and 1400 mg/kg bw/ day (EPA-US, 2009) during a two week premating period, gestation, parturition, and lactation, while male rats remained unexposed. The authors examined the proportion of pregnancies, duration of parturition, live and dead pups, and complete and partial litters, pup body weights, and maternal (during parturition and postpartum) and pup (PND 4 and 21) survival.

1.2.1 Adverse effects of BENPAT constituent DPPD on sexual function and fertility

In a Reproductive/ Developmental Toxicity Screening Test, conducted according to OECD TG 421, gastric intubation of DPPD at 50 and 300 mg/kg bw/d resulted in a statistical significant prolonged gestation compared to control animals. The number of pups born, delivery index, number of live pups, birth index, and live birth index on day 0 of lactation were dose-dependently decreased, but data were not statistical significantly different from controls. On day four of lactation, the number of live pups and viability were decreased in DPPD-treated animals (300 mg/kg bw/d), compared to controls. There were no changes observed in litter weights and body weights of pups on days 0 and 4 of the lactation. Furthermore, gross examinations revealed no external or internal abnormalities in pups.

Each of the aforementioned none-guideline studies with DPPD from the 1950s identified a prolonged pregnancy in DPPD treated rats compared to controls. Ashe (1956) indicated a possible increase in gestation time in females treated with DPPD at 31 and 103 mg/kg bw/d. However, the author reported approximate data because timing of conception was not accurately determined for all animals (EPA-US, 2009). In the same study, dose-related offspring mortality was shown and pups died primarily at birth. Mean number of live pups at birth and the ratio of the numbers of litters weaned divided by the number of pregnant dams were decreased at doses of DPPD at 31 and 103 mg/kg bw/d. It was reported that the "affected offspring were deeply cyanotic" and the authors concluded that "foetal deaths were due to anoxia resulting from partial or complete placental separation at term with inadequate uterine contraction" (EPA-US, 2009). Ames and colleagues identified prolonged gestations in rats treated with DPPD at doses equal to and higher than 55 mg/kg bw/d. Furthermore, significantly increased pup mortality, compared to control animals, was observed on and before PND 3, and most of the deaths occurred during parturition (DPPD≥11 mg/kg bw/d). Mean litter size from all DPPD treated females was decreased. Investigations of (Oser and Oser, 1956) revealed a significantly prolonged gestation in all DPPD treated females (DPPD ≥22 mg/kg bw/d) accompanied with a delayed parturition, "in some cases as much as 6 days" (Oser and Oser, 1956). The stillbirth index was significantly increased from dams treated

with DPPD at concentrations ≥ 22 mg/kg bw/d. Number of litters born complete were dose-dependently reduced in DPPD treated rats, compared to controls. The mean body weight of pups born dead or in utero from DPPD exposed females was 10 to 20 % increased (no dose-response), whereas the mean body weight of pups born alive was increased (DDPD: 22 mg/kg bw/d), or reduced (350 and 1400 mg/kg bw/d).

During the reproductive/ developmental toxicity screening test, gastric intubation of DPPD (8, 50, or 300 mg/kg bw/d) did not result in effects on the number of mated pairs, number of copulated pairs, copulation index, number of fertile males, or fertility index. There were no effects on the length of estrus cycle, pairing days until copulation, number of corpora lutea, number of implantations, implantation index, and number of pregnant females.

Ashe (1956) reported a possible slight decrease in fertility of females treated with DPPD at approx. 31 and 103 mg/kg/bw day, compared to controls (EPA-US, 2009). However, (Oser and Oser, 1956) did not identify effects on fertility of DPPD treated females (approx. 22, 88, 350, and 1400 mg/kg bw/day), shown by a normal proportion of pregnancies. From the study of Ames and colleagues (Ames et al., 1956) no data were available concerning adverse effects on sexual function and fertility.

1.2.2 General toxicity from studies performed with BENPAT constituent DPPD

Gastric intubation of DPPD at 8, 50, and 300 mg/kg bw/day did not reveal effects on male or female body weights, body weight gains, and food consumption**.** In males, neither death nor clinical toxicity was observed. Among females, two rats were observed with piloerection, hypothermia, and pale skin on day 23 of pregnancy. One of these two females died and the other was sacrificed due to dystocia on day 23 of pregnancy. Another female with piloerection and pale skin delivered only three live pups. This female was sacrificed on day 1 of lactation due to total litter loss. Gross pathological findings of two females that died or were sacrificed on day 23 of pregnancy revealed "hemorrhage in the lumen of the uterus, incomplete retention and red colour in the lung, and dark red medulla and hardness on the kidney in both animals; hydrothorax in the thoracic cavity, attachment of red content in mucosa of the glandular stomach and recessed area, or red spots in the duodenum in either animal. In the histopathological examination, slight hemorrhage in the endometrium, and very slight edema, very slight foam cell accumulation in alveolus, and very slight capillary fibrinous thromboses in the lung were observed in the two females. The histopathological examination revealed no toxicological effects in other males and females" (Matsumoto et al., 2013).

Possible treatment-related effects on maternal mortality in females fed with 31 and 103 mg/kg bw/day pure DPPD were observed by Ashe (1956) and all deaths occurred during parturition. Furthermore, dams of all DPPD-treated groups, but not controls, hemorrhaged abnormally during delivery. The authors reported that surviving dams were severely anemic for many weeks. However, no data were presented to expound these effects. Ashe conducted gross and microscopic examinations of rats that died during labor and of those that failed to become pregnant. However, the authors did not specify the test material (pure DPPD, commercial DPP, or contaminants) (EPA-US, 2009) and therefore data show a high uncertainty and were not considered for assessment. During another study maternal mortality was significantly increased in dams treated with DPPD ≥275 mg/kg bw (Ames et al., 1956). Also in this study, females died mainly during labor and signs of difficult parturition were occasionally observed, e.g. vaginal bleeding or prolapse of the uterus. (Oser and Oser, 1956) also observed maternal mortality, with a significant increase in highest dose (1400 mg/kg bw/day), compared to control animals. The post-partum survival of females was diminished at levels of DPPD ≥350 mg/kg bw/d (not statistically different from controls). The authors histopathological examined the pituitary of DPPD-fed rats that died in parturition (GD 24 and 25, N=5). Investigations did not give any evidence of organic injury of the pituitary between treated animals and controls.

In summary, adverse effects of DPPD on fertility (dystocia) are considered to be due to prostaglandin inhibition.

1.3 **Repeated dose toxicity studies with BENPAT – for information only**

Table 2: Summary table of animal studies on repeated dose toxicity

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, route of exposure, dose levels**	**Results**
Oral 4-week dietary study; similar to OECD TG 407 **Key study** GLP-compliant Rat, Fischer 344 8 animals/sex/dose group sacrificed after 28 days of exposure; for 6 male and female control and high dose animals, a treatment-free period of 2 weeks followed (AHF, 1994)	BENPAT, CAS 68953-84-4; dietary doses of 0, 7.5, 30 and 120 mg/kg bw/d Purity: no data	No compound related deaths occurred **120 mg/kg bw/d** ↓ body weight gain in ♀, started after first week administration; reduction recovered within first treatment-free week ↓ food consumption in ♂ by day 12, which continued until day of sacrifice & ↓ food consumption in ♀ with a plateau effect at the end of 2 week exposure-free period ↑ mean corpuscular volume (MCV) in ♂ & ♀ ↓ mean corpuscular hemoglobin concentration (MCHC) in ♂ & ♀; recovery trend by being of lesser degree of significance at 42 days During the recovery phase, ↑ reticulocyte count in ♂ & ♀ ↑ total bilirubin in ♂ at day 28, levels returned to control values during recovery ↑ cholesterol in ♂ & ♀ at day 28, finding recovered completely in ♂, degree of significance was reduced during recovery in ♀ ↑ total protein and albumin in ♂ during recovery ↑ absolute & relative liver weights in ♂ & ♀ at day 28, finding completely recovered by day 42 ↑ relative kidney weights in ♂ & ♀ at day 28, by day 42 this finding remained significant in males, but not in females. ↑ relative heart and splenic weights in ♀ at 28 days, finding showed a recovering trend by day 42 ↓ mean renal cell nuclei labelling (PCNA) index in ♀, during recovery at 42 days, the labelling index had increased significantly over control animals ↑ mean urothelial cell nuclear (PCNA) labelling index in ♂ & ♀ at 28 days **30 mg/kg bw/d** ↓ food consumption in ♂ by day 26 & in ♀ ↓ mean corpuscular hemoglobin concentration (MCHC) in ♀ ↑ total bilirubin in ♂ at day 28, levels returned to control values during recovery ↑ mean hepatocellular nuclear labelling (PCNA) index ♂ at day 28 ↑ mean urothelial cell nuclear labelling (PCNA) index in ♂ & ♀ at 28 days **7.5 mg/kg bw/d** reduction of food consumption in ♂ by day 26 ↑ mean hepatocellular nuclear labelling (PCNA) index ♂ & ♀ at day 28 ↑ mean urothelial cell nuclear labelling (PCNA) index in ♀ at 28 days

		There was no pertinent or compound-related gross finding in either gender: Most changes observed during microscopic evaluation were not compound related LOAEL of 7.5 mg/kg bw/d should be derived, based on urothelial cell proliferation (occurring) in different studies
Oral 52 week dietary study, none-guideline study **<u>Supporting study</u>** GLP-compliant Rat, Fischer 344 N=6 animal/sex/dose sacrificed after 38 weeks; 20 animals/ sex/dose (control: 12 animals per sex) sacrificed after 52 weeks; 6 animals/ sex/dose exposure-free for 12 weeks after 52-week administration period (AHF, 1996)	BENPAT, CAS 68953-84-4; dietary doses of 53, 310 and 1900 ppm (corresponding to nominal doses of 0, 3.3, 20 and 120 mg/kg bw/d of the test substance)	No compound-related deaths or clinical findings occurred in this study. **<u>120 mg/kg bw/d</u>** ↓ body weight gain in ♀ (-18 %), including the recovery period (-10 %, not significant) ↓ body weight in ♀ & ♂ during exposure period, body weight of ♀ lower compared to controls during exposure-free period ↓ food consumption in ♂ (weeks 16-30 and at 52 weeks) & ♀ (started with week 12, remained reduced during recovery) ↑ MCV in ♀ & ♂ at week 52, recovery during exposure-free period ↓ MCHC in ♀ & ♂ at week 52, recovery during exposure-free period ↓ red blood cell count (RBC) in ♂ at week 52, recovery during exposure-free period ↓ hemoglobin (HGB) in ♂ & ♀, recovery during exposure-free period ↑ total bilirubin in ♂ at 52-week, returned to control values during exposure-free period; (consistently) ↓ total bilirubin in ♀ at 38 & 52 weeks ↑ urinary bilirubin in ♂ at 38 week ↑ relative liver weight , ↑ relative kidney weight in ♂ & ♀; ↑ relative heart weight in ♀; recovered during exposure-free period ↑ relative spleen weight in ♂ & ♀, still ↑ in ♂ during exposure-free period ↑ urothelial cell nuclei labelling index in ♂ & ♀, still increased during exposure-free period **<u>20 mg/kg bw/d</u>** ↑ urothelial cell nuclei labelling index in ♂ & ♀; still <u>increased</u> during exposure-free period ↑ relative liver weight & ↑ relative heart weight in ♀; recovered during exposure-free period **<u>3.3 mg/kg bw/d</u>** ↑ urothelial cell nuclei labelling index in ♂; still <u>increased</u> during exposure-free period

Three oral repeated dose toxicity studies performed with BENPAT are available. An oral 3-week gavage study was conducted as a range-finding study. Furthermore, a 4-week dietary study and a 52-week chronic dietary study are available. The two latter studies were identified as key studies by the registrants and are summarised below. Repeated dose toxicity studies with other exposure routes (dermal, inhalation) are not available.

1.3.1 Oral 4-week dietary study

The study did not adhere to any guideline. However, many elements were similar to OECD TG 407 and the study was performed in accordance with GLP principles. The DS considers this study as the key study on specific target organ toxicity after repeated exposure of BENPAT. Male and female Fischer 344 rats were exposed via diet to BENPAT doses of 0, 7.5, 30 and 120 mg/kg bw/d. After 28 days of exposure, eight animals/sex/dose group were killed. For six male and female control and high dose animals, a treatment-free period of two weeks followed. At sacrifice, hematology, clinical chemistry, urinalysis, and complete necropsies were performed. The absolute and relative weights of selected tissues (liver, kidneys, pituitary, uterus, heart, brain, spleen, thyroids, adrenals, testes, and ovaries) were recorded; sampled tissues were processed and subjected to macroscopic evaluation. Slides of liver, kidneys, and urinary bladder were subjected to immunohistochemical staining for proliferating cell nuclear antigen (PCNA), but not for standard H&E staining.

Results:

The following effects were observed: increased liver and kidney weights in mid-dose females and in high-dose males and females; increased heart and spleen weight and decreased body weight in high dose females (with reduced food consumption), but no pertinent or compound-related gross findings. Increased cell division for liver and decreased cell division for kidney cells as evidenced by PCNA staining: at 28 days, there was a significant increase of the mean hepatocellular PCNA labelling index in males and females of the low dose ($p<0.01$). High dose females exhibited a decrease in the mean renal cell PCNA labelling index ($p<0.05$).

Reversible hematological signs (including significantly ($p<0.01$) increased mean corpuscular volume, and significantly ($p<0.01$) decreased mean corpuscular hemoglobin concentration in high dose animals of both sexes) and clinical chemistry changes (including significantly increased blood bilirubin at high- ($p<0.01$) mid- ($p<0.05$) dose animals and significantly increased cholesterol levels in high dose animals ($p<0.01$).

At 28 days, mean urothelial PCNA indices of the low and mid dose groups were increased (statistically significant ($p<0.05$) only for low-dose females), but heavily decreased in high dose animals.

1.3.2 Oral 52-week dietary study

The study did not adhere to any guideline; however, it was performed in accordance with GLP principles. Groups of male and female Fischer 344 rats received dietary levels of 0 (control), 53, 310 and 1900 ppm of the test substance (corresponding to nominal doses of 0, 3.3, 20 and 120 mg/kg bw/d of the test substance). Six animal/sex/dose were sacrificed after 38 weeks; 20 animals/sex/dose (control: 12 animals per sex) were sacrificed after 52 weeks. Another six animals/sex/dose were kept exposure-free for 12 weeks after the 52-week administration period. Clinical observations, body weights, and food consumption were monitored. At sacrifices (after 38, 52 and 64 weeks) hematology, clinical chemistry, urinalysis, and complete necropsies were performed and the absolute and relative weights of selected tissues (liver, kidneys, pituitary, heart, spleen, thyroids, adrenals, brain, uterus, ovaries and testes) were recorded. All sampled tissues were processed and subjected to microscopic evaluation. Slides of liver, urinary bladder, and kidneys were also subjected to immunohistochemical staining for proliferating cell nuclear antigen (PCNA).

Results:

During the 52-week dietary study, no compound related deaths or clinical findings occurred. Compared to controls, body weight gains were non-significantly and reversibly decreased in high dose males and significantly and non-reversibly decreased in high dose females. Relative kidney and heart weights were increased at all sacrifices in the high dose animals.

The mean corpuscular volume (MCV) was statistically significantly increased in male and female high-dose animals ($p<0.01$ for both sexes), whereas the mean corpuscular hemoglobin concentration (MCHC) was decreased at 38 and 52 weeks of the high dose groups (statistically significant at 52 weeks in both sexes, $p<0.01$). At week 52, red blood cell counts (RBC) were statistically significantly decreased (-13.2 %) in high-dose males ($p<0.01$) and hemoglobin values were statistically significantly decreased (-10.7 %) in high dose

males ($p<0.01$) and females ($p<0.05$). Hematological parameters returned to control values in the treatment-free period. Liver and splenic weights were also increased at 38 and 52 weeks. Extramedullary erythropoiesis was evident in these organs at the highest dose tested in both male and female animals indicating the presence of a chronic macrocytic anemia. In the high dose males only, total (blood) bilirubin was increased at both intervals, accompanied with increases in urinary bilirubin (at 38 weeks only). There was also an increase in serum cholesterol in high dose groups of both sexes and at both time intervals. Urothelial cell PCNAs were significantly increased in a dose- and time-related manner at all sacrifices (weeks 38, 52 and 64). PCNAs in liver and kidneys were not significantly different from controls. Urinary pH remained constant through all sacrifices and dosages. There were no compound-related findings in any of the organs or tissues examined microscopically by H&E staining, except for the evidence of extramedullary erythropoiesis in the spleen and liver of high doses animals of both sexes at the 52-week sacrifice. No indication on treatment-related carcinogenic activity was found in the study. Main chronic toxic effects were a mild chronic macrocytic anemia and increases in urinary bladder cell proliferation. At the end of the study, no compound-related pre-neoplastic changes or neoplasms were present.

In summary, significant anemic effects (reductions in RBC and hemoglobin) were observed at the high dose level only. Increases in blood bilirubin in high dose males only indicated hemolysis as a possible cause of the loss of erythrocytes. Extramedullary hematopoiesis in the liver and the spleen indicates compensatory erythropoiesis and was consistently observed in high dose males and females.

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