JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Aminoparathion: A Highly Reactive Metabolite of Parathion. 1. Reactions with Polyphenols and Polyphenol Oxidase

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Spiking of tomato and apple fruits with parathion at different levels of about 1−4 mg/kg irradiation and under simulated sunlight conditions resulted in nearly complete photodegradation within 13 h, but extractable parathion degradation products could not be found in any case. However, after irradiation of an unrealistically spiked apple (134 mg/kg) different photoproducts including aminoparathion (AP) were detectable by HPLC, proving that the hitherto postulated photochemistry of parathion indeed takes place in the fruit cuticle environment. Besides the photoreduction pathway it was shown for the first time that AP is also easily formed by reduction of the primary photoproduct nitrosoparathion with thiols (cysteine, glutathione), while ascorbic acid only leaves hydroxylaminoparathion. In the presence of polyphenols, AP was effectively bound to quinone intermediates formed by both silver oxide and polyphenol oxidases. For pyrocatechol, a disubstituted *o*-quinone derivative could be isolated as a dark red addition product and structurally be elucidated. However, in the presence of caffeic acid, catechol, naringin, and quercetin, respectively, insoluble dark colored polymers precipitated within 48 h, while in the supernatants AP was not detectable any more. Polymer-bound and nonextractable AP was proven by transesterification with sodium ethoxide releasing *O*,*O*,*O*-triethyl thiophosphate which was determined by GC. Additionally, AP itself was a substrate for polyphenol oxidases, resulting in a quinone imine intermediate which in turn reacted with excessive AP yielding deep red colored di- and trimerization products.

KEYWORDS: Parathion; nitrosoparathion; hydroxylaminoparathion; aminoparathion; polyphenols; polyphenol oxidase; bound residues

INTRODUCTION

The organophosphorus insecticide parathion (**1**, **Figure 1**) is known to be efficiently photoreduced in plant cuticle environments, yielding nitroso- (**2**), (hydroxylamino)- (**3**), and aminoparathion (**4**) (*1*−*4*). Due to their high reactivity, it is rather difficult to detect the reduction products both during in vitro experiments and, for example, on fruits. However the photoreduction was unequivocally deduced from the formation of azoxyparathion (**5**) and azoparathion (**6**) (**Figure 1**) as condensation products both in model experiments and on isolated fruit cuticles (*3*). On the basis of the easy addition of nitrosoparathion to unsaturated fatty acids (*3*−*6*), also the formation of fruit cuticle-bound residues of parathion was recently shown (*7*). Directly correlated to sunshine, parathion was readily degraded during a field experiment on apples, while fruit cuticle-bound residues were clearly determined by ELISA (*8*).

Besides photoreduction, another way is the reduction of the phenylnitro group by the plant metabolism itself. Suzuki and Uchiyama determined the formation of **2**−**4** in spinach homogenate fortified with **1** (*9*−*11*). Due to this fact and additionally due to hydrolysis and oxidation reactions, it is not astonishing

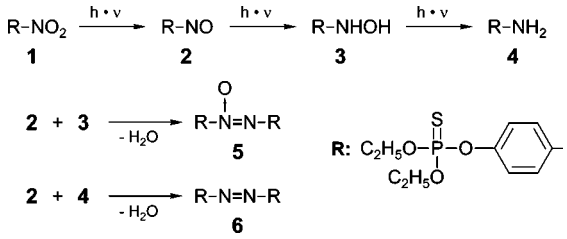

Figure 1. Products formed by the photoreduction of parathion (**1**) (nitrosoparathion (**2**), (hydroxylamino)parathion (**3**), and aminoparathion (**4**)) and the condensation products azoxyparathion (**5**) and azoparathion (**6**), respectively.

that **1** cannot be recovered in high yields from spiked products as juices after a certain period of time (*12*−*14*). Spiking of grapes, apples, and tomatoes with **1** and then processing into products such as wine, cider, and ketchup resulted in nearly complete degradation, wheras only small residues of **4** could not explain the disappearance of **1** (*12*−*14*). For instance, about 85% of **1** added to tomatoes (fortification level: 25 μg/g) was lost during the processing steps, whereby only 1.7% of the inital **1** were detected together with low levels of **4** and *p*-nitrophenol after 6 months of storage (*14*).

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10.1021/jf051520m CCC: $30.25
Published on Web 10/15/2005

Derivatives of nitrosobenzene or aniline should also be capable by polyphenols widely present in plants, mostly higher concentrated in fruit peels as compared to fruit flesh (*15*). For example, quercetin and phloretin glycosides appear in apple peels in the range of 0.2−5 mg/g (*16*), and about 0.5 mg/g of quercetin was found in tomato peels (*17*). Other polyphenolic compounds such as chalconaringenin, naringenin, naringenin-7-glucoside, and *m*- and *p*-coumaric acids, identified in the fruit cuticles of tomato cultivars, reached amounts of up to 6% (total phenolics 7−14%) of the cuticle mass in mature fruits (*18*).

Polyphenols are also substrates of the so-called enzymatic browning (*19*, *20*). By the action of polyphenol oxidases quinone intermediates are formed, which in turn rapidly induce phenol polymerization processes affording brown colored reaction products. Additionally it is well-known that not only phenols but also proteins are involved in these complex processes (*19*−*21*). The nucleophilic amino or mercapto groups of proteins are quite suitable reaction partners of quinones.

Therefore, the aim of the present research was to study if the reactivity of the parathion reduction product aminoparathion is a reason for the disappearance of parathion in processed fruits and vegetables (*12*−*14*) and if phenol-bound or conjugated residues of aminoparathion are formed.

MATERIALS AND METHODS

Reagents. The solvents used were of analytical grade (Merck, Darmstadt, Germany) and distilled before use. Water was purified by a Milli-Q 185 plus water purification system (Millipore, Bedford, MA). Polyphenol oxidase was purchased from Worthington (Lakewood, NJ). If not otherwise described, all chemicals used were obtained from Fluka (Steinheim, Germany).

Instrumentation and Equipment. *High Performance Liquid Chromatography (HPLC).* A HP 1100 HPLC system was used, consisting of a degasser, column oven, autosampler, gradient pump, and diode array detector (DAD) module (Agilent, Waldbronn, Germany). Data aquisition and processing was performed by HP ChemStation software (rev A.04.02) with DAD detection wavelengths 235 nm (spectral bandwidth (SBW) 8 nm), reference 500 nm (SBW 100 nm) and 505 nm (SBW 8 nm), and reference 600 nm (SBW 40 nm). A reversed phase analytical column (5 μm Eurospher 100-C18 250 × 3 mm, Knauer, Berlin, Germany) including a precolumn (Nucleosil 5 μm C_{18}, 5 × 3 mm, Knauer, Berlin, Germany) was used at 25 °C. The mobile phase consisted of 20 mM phosphate buffer pH 4.0 (A) and methanol (B): gradient % B (*t* (min)): 60 (0)−80 (12)−90 (20)−90 (28)−60 (31)−60 (35); flow rate 0.5 mL/min; injection volume 10 μL.

High Performance Liquid Chromatography/Mass Spectrometry (LC/MS). LC/MS analyses were performed on a HPLC system (as described above), coupled to a VG platform II quadrupole mass spectrometer (Micromass, Manchester, U.K.) equipped with an electrospray interface (ESI). For data acquisition and processing, MassLynx 3.2 software was used. The mobile phase consisted of 10 mM ammonium formate buffer (pH 4.0) and methanol. The other conditions were as described above. MS parameters: ESI+; source temperature 120 °C; capillary 3.0 kV; HV lens 0.5 kV; cone 55 V. For LC analysis, the MS was operated in full-scan mode (*m*/*z* 200−1000). For accurate mass determination (*22*), data were collected in the multichannel acquisition (MCA) mode with 128 channels per *m*/*z* unit using 11 scans (5 s) with 0.1 s reset time. The resolution was 1530 (10% valley definition). The samples were dissolved in ammonium formate buffer (0.1 mol/L)/methanol (1 + 1; v/v) containing poly(ethylene glycol) 400 (0.1 μg/μL), poly(propylene glycol) 425 (0.1 μg/μL). or poly(propylene glycol) 725 (0.1 μg/μL), respectively, as reference material; the sample concentrations were similar to that of the reference standards. The solutions were introduced into the ESI source (source temperature 80 °C) at a flow rate of 5 μL/min. With *m*/*z* scan ranges 220−350, 325−420, 440−604, and 555−720, five reference peaks could be used for mass calibration.

Preparative HPLC. The preparative HPLC system comprised of two HD2-200 pumps (Kronlab, Sinsheim, Germany), for gradient elution controlled by PrepCon (Preparative HPLC Pump Control, ver 3.0, SCPA GmbH, Stuhr, Germany), combined with an A0293 variable wavelength monitor (Knauer, Berlin, Germany) and a column (Nucleosil RP18, 250 × 20 mm, 7 μm, Kronlab, Sinsheim, Germany) including a precolumn (Nucleosil RP18, 50 × 20 mm, 7 μm). Data aquisition was performed by a C-R3A Chromatopac integrator (Shimadzu, Duisburg, Germany). The mobile phase consisted of 10 mM ammonium formate buffer (pH 4.0) and methanol at a flow rate of 18 mL/min (gradient as described above for analytical HPLC). The injection volume was 2 mL.

Gas Liquid Chromatography (GLC/FID). GLC was performed using a PE8600 gas chromatograph (Perkin-Elmer, Rodgau, Germany) equipped with a flame ionization detector (FID) and a J & W (Folsom, CA) fused silica capillary column (30 m × 0.32 mm) wall-coated with DB5 (0.25 μm film thickness). The oven temperature was programmed to start at 100 °C, followed by an increase of 8 °C/min to 270 °C, held for 10 min. Injector and detector temperatures were set at 270 °C; injection (1 μL) was in the split mode (1:5). Helium was used as the carrier gas with a column head pressure of 80 kPa.

Gas Liquid Chromatography/Mass Spectrometry Analysis (GLC/MS). GLC/MS was performed on a Finnigan MAT (Bremen, Germany) Ion Trap 800, 70 eV EI, coupled to a PE8420 gas chromatograph (Perkin-Elmer, Rodgau, Germany) equipped a J & W (Folsom, CA) fused silica capillary column (30 m × 0.25 mm) wall-coated with DB5-MS (0.25 μm film thickness). Oven temperature program was given above. Helium was used as the carrier gas with a column head pressure of 80 kPa.

UV Spectroscopy. UV spectra were measured with a Perkin-Elmer UV/vis spectrometer Lambda 2 (Perkin-Elmer, Rodgau, Germany).

IR Spectroscopy. IR spectra were measured with a Nicolet Avatar 320 ESP spectrometer (Nicolet, Offenbach aM, Germany) by means of the ATR technique.

Nuclear Magnetic Resonance Spectroscopy (NMR). NMR spectra were recordered on Varian Unity Inova-300 and Varian Unity Inova-500 spectrometers (Varian, Darmstadt, Germany) at 300 and 500 MHz (^{1}H) as well as 75 and 125 MHz (^{13}C), respectively, at room temperature. Chemical shifts (δ) are given in ppm relative to tetramethylsilane; s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet, with dd, dt, dq, and ddd denoting a combination.

Irradiation Equipment. The irradiation experiments in solution were performed using a metal halogenide lamp SOL 500 (Dr. K. Hönle AG, Planegg, Germany) with a cutoff filter WG295 (Schott, Mainz, Germany) and water-cooled quartz cuvettes. Fruits were irradiated employing a suntest CPS+ (Heraeus, Kleinostheim, Germany) with xenon lamp (250 W/m^2, black standard temperature 35 °C, air-cooling).

Synthesis of *O*,*O*-Diethyl *O*-(4-Aminophenyl) Thiophosphate (Aminoparathion, 4). Sodium *tert*-butoxide (25 mmol, 2.35 g) and 4-aminophenol (20 mmol, 2.18 g) were supended in 2-butanone (100 mL) and, under stirring, heated to 70 °C. *O*,*O*-Diethyl chlorothiophosphate (25 mmol, 4.72 g) dissolved in 2-butanone (20 mL) was added dropwise, and the mixture was held at 70 °C for 2 h. After further stirring at ambient temperature for 14 h, the reaction mixture was poured into water (100 mL) and the crude product was extracted with diethyl ether (50 mL, twice). The combined extracts were washed with sodium hydroxide solution (0.02 mol/L, 400 mL) and brine (300 mL), dried over sodium sulfate, and evaporated. The oily residue was purified by preparative HPLC, yielding 3.72 g (71.3%) of **4** as clear and colorless oil: UV/vis (methanol) λ_{max} (nm) (log ϵ) 203 (4.35), 238 (4.02), 292 (3.24); GLC/MS (EI, 70 eV) *m*/*z* = 261 (65%), 233 (17%) [M − C_2H_4]$^+$, 205 (24%) [M − C_2H_4 − C_2H_4]$^+$, 187 (5%), 125 (100%), 109 (63%), 108 (61%), 97 (39%), 80 (65%); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm) 6.80 (m; 2 H), 6.51 (m; 2 H), 5.01 (4-NH_2; s; 2 H), 4.12 (dq; 4 H; J = 7.1/9.9 Hz), 1.25 (t; 6 H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm) 146.28 (d; J = 1.2 Hz), 140.53 (d; J = 7.8 Hz), 121.18 (d; J = 4.5 Hz), 114.25 (d; J = 1.2 Hz), 64.62 (d; J = 6.0 Hz), 15.74 (d; J = 7.1 Hz).

Synthesis of *O*,*O*-Diethyl *O*-(4-Nitrosophenyl) Thiophosphate. (Nitrosoparathion, 2). Aminoparathion (**4**) (9.6 mmol, 2.52 g) was dissolved in 100 mL of methanol and cooled to −18 °C. To this solution was added a cooled (−18 °C) solution of *m*-chloroperbenzoic acid (70%, 19.3 mmol, 4.76 g) in 15 mL of methanol stepwise, while stirring.

After 15 min 200 mL of water was added and the product extracted twice with 50 mL of diethyl ether. The organic layer was washed with 300 mL of sodium carbonate solution (0.1 mol/L) and 300 mL of brine. Purification performed by column chromatography on silica gel (25 g) with petroleum ether/diethyl ether (1:1 v/v) as eluent yielded 637 mg (24.0%) of **2** as a clear green oil: ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm) 8.03 (m; 2 H), 7.51 (m; 2 H), 4.25 (dq; 4 H; J = 7.1/10.3 Hz), 1.31 (t; 6 H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm) 163.94 (s), 156.01 (d; J = 7.2 Hz), 121.90 (d; J = 5.1 Hz), 123.32 (s), 65.58 (d; J = 6.0 Hz), 15.72 (d; J = 7.2 Hz).

Synthesis of *O,O*-Diethyl *O*-(4-Hydroxyaminophenyl) Thiophosphate. (Hydroxylaminoparathion, 3). To a solution of **2** (0.07 mmol, 19.3 mg) in 3 mL of methanol was added a solution of ascorbic acid in water (15.4 mg/mL), and the mixture was shaken for 5 min. Purification performed by preparative HPLC yielded 6.0 mg (30.9%) of **3** as a clear green oil: UV/vis (methanol) λ_{max} (nm) (log ϵ) 238 (3.96), 285 (3.08); IR (ATR) ν (cm^{-1}) 3279 (w), 2982 (w), 2929 (w), 2907 (w), 1739 (w), 1597 (w), 1501 (m), 1442 (w), 1390 (w), 1291 (w), 1220 (w), 1200 (m), 1160 (m), 1099 (w), 1013 (s), 918 (s), 816 (s), 781 (s), 721 (w), 689 (w); accurate mass (mean of 8 measurements ± standard deviation) m/z = 278.0625 ± 0.0007 [M + H]$^+$ (calcd m/z 278.0616 for $C_{10}H_{17}NO_4PS$); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm) 6.96 (m; 2 H), 6.80 (m; 2 H), 4.14 (dq; 4 H; J = 7.1/10.0 Hz), 1.26 (t; 6 H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm) 149.54 (s), 143.07 (d; J = 7.8 Hz), 120.86 (d; J = 4.2 Hz), 113.75 (s), 64.76 (d; J = 5.7 Hz), 15.75 (d; J = 7.2 Hz).

Synthesis of *O,O,O*-Triethyl Thiophosphate (TETP). According to the method described by Wettach et al. (*7*), the preparation of TETP was performed from sodium ethoxide (11 mmol: 0.26 g of Na in 6 mL of ethanol) and *O,O*-diethyl chlorothiophosphate (8 mmol, 1.50 g). However, instead of heating the mixture was stirred at ambient temperature for 14 h. Then the reaction mixture was poured into water (50 mL) and extracted three times with 20 mL of diethyl ether. After being dried over sodium sulfate and evaporation, without further purification, 1.24 g (78.2%) of pure TETP was obtained as colorless clear liquid: GLC/MS (EI, 70 eV) m/z = 198 (76%), 170 (9%) [M − C_2H_4]$^+$, 153 (8%), 143 (19%), 142 (17%) [M − C_2H_4 − C_2H_4]$^+$, 126 (26%), 121 (80%), 115 (50%), 114 (52%), 109 (33%), 97 (61%), 93 (72%), 81 (28%), 65 (100%).

Synthesis of *O*-(4-{[6-({4-[(Diethoxyphosphorothioyl)oxy]phenyl}-amino)-3,4-dioxo-1,5-cyclohexadien-1-yl]amino}phenyl) *O,O*-Diethyl Thiophosphate (7). Aminoparathion (**4**) (0.54 mmol, 140 mg) and pyrocatechol (0.54 mmol, 59 mg) were dissolved in dried diethyl ether (6 mL), and silver oxide (1.07 mmol, 248.6 mg) was added. After the mixture was stirred for 30 min at ambient temperature, the solids were filtered off and the solvent was evaporated. The crude residue was purified by preparative HPLC and yielded 9.3 mg (5.5%) of 7 as a dark red, strongly viscous liquid: UV/vis (methanol) λ_{max} (nm) (log ϵ) 224 (4.24), 262 (4.13), 303 (4.14), 433 (3.62); IR (ATR) ν (cm^{-1}) 3277 (w), 2981 (w), 2916 (w), 2870 (w), 1741 (w), 1608 (m), 1585 (m), 1557 (m), 1504 (s), 1491 (s), 1407 (m), 1391 (m), 1336 (w), 1291 (w), 1197 (s), 1161 (s), 1097 (m), 1012 (s), 919 (s), 818 (s), 791 (s), 756 (m), 742 (m), 708 (m); LC/MS (ESI+) m/z (relative intensity) = 627.07 (100%), 628.13 (29.2%), 629.12 (15.2%), 630.11 (3.8%), 631.10 (1.0%); accurate mass (mean of 6 measurements ± standard deviation) m/z = 627.1146 ± 0.0006 [M + H]$^+$ (calcd m/z 627.1154 for $C_{26}H_{33}N_2O_8P_2S_2$); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm) 7.22 (m; 8 H), 5.75 (s; 2 H), 4.21 (dq; 8 H; J = 7.0/10.2 Hz), 1.30 (dt; 12 H; J = 0.6 Hz); ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm) 147.25 (d; J = 7.8 Hz), 121.56 (d; J = 4.8 Hz), 97.24 (s), 65.04 (d; J = 5.7 Hz), 15.73 (d; J = 6.9 Hz). Note that some carbon signals are not detectable due to rapid keto−enol tautomerisms of the *ortho*-quinoid ring system.

Synthesis of *O*-[4-({6-[(Diethoxyphosphorothioyl)oxy]-3-imino-4-oxo-1,5-cyclohexadien-1-yl}amino)phenyl] *O,O*-Diethyl Thiophosphate (8), *O*-(2-Amino-3-oxo-3*H*-phenoxazin-7-yl) *O,O*-Diethyl Thiophosphate (9), and *O*-(4-{[3-Amino-4-({4-[(diethoxyphosphorothioyl)-oxy]phenyl}amino)-6-oxo-2,4 -cyclohexadien-1-ylidene]amino}phenyl) *O,O*-Diethyl Thiophosphate (10). To a solution of polyphenol oxidase (PPO) in phosphate buffer pH 6.5 (25.5 mg/500 mL = 74460 U/L; PPO activity, 1460 U/mg of dry weight) a methanolic solution of 4 (93.3 mg/5 mL, 0.36 mmol) was added. The mixture was stirred vigorously for 70 h and extracted with diethyl ether (200 mL, four times). After drying over sodium sulfate and solvent evaporation, 8−10 were isolated by preparative HPLC. Whereas 8 was only stable in solution and completely decomposed during isolation, 9 and 10 were individually obtained in yields of 8.1 mg (11.9%) and 4.6 mg (6.2%), respectively.

*Compound **8***: UV/vis (methanol) λ_{max} (nm) 233, 300, 486; LC/MS (ESI+) m/z (relative intensity) = 535.14 (100%), 536.10 (25.2%), 537.06 (13.3%), 538.06 (2.8%), 539.06 (0.7%); accurate mass (mean of 5 measurements ± standard deviation) m/z = 535.0902 ± 0.0016 [M + H]$^+$ (calcd 535.0891 for $C_{20}H_{29}N_2O_7P_2S_2$).

*Compound **9***: red crystals, UV/vis (methanol) λ_{max} (nm) (log ϵ) 237 (4.06), 437 (3.99); IR (ATR) ν (cm^{-1}) 3445 (m), 3336 (m), 2980 (w), 2931 (w), 1712 (w), 1650 (w), 1584 (s), 1568 (s), 1504 (m), 1466 (s), 1449 (s), 1415 (s), 1400 (m), 1367 (m), 1312 (w), 1287 (m), 1245 (s), 1181 (s), 1163 (s), 1139 (m), 1118 (s), 1040 (m), 1012 (s), 959 (s), 897 (s), 868 (s), 852 (s), 843 (s), 812 (s), 793 (s), 765 (s), 741 (s), 696 (s), 659 (s); LC/MS (ESI+) m/z (relative intensity) = 381.07 (100%), 382.11 (19.1%), 383.08 (7.3%), 384.09 (1.2%); accurate mass (mean of 5 measurements ± standard deviation) m/z = 381.0667 ± 0.0007 [M + H]$^+$ (calcd 381.0674 for $C_{16}H_{18}N_2O_5PS$); ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm) 7.73 (d; 1 H; J = 8.8 Hz), 7.32 (dd; 1 H; J = 2.5/1.7 Hz), 7.21 (ddd; 1 H; J = 1.5 Hz), 6.79 (NH_2; s; 2 H), 6.37 (s; 1 H), 6.35 (s; 1 H), 4.24 (dq; 4 H; J = 7.1/10.3 Hz), 1.31 (dt; 6 H; J = 0.7 Hz); ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm) 180.45 (s), 149.61 (d; J = 7.5 Hz), 148.77/148.07/147.49 (s), 142.36 (d; J = 1.0 Hz), 131.57 (d; J = 1.8 Hz), 129.08 (d; J = 1.0 Hz), 118.53 (d; J = 5.1 Hz), 108.56 (d; J = 5.4 Hz), 103.99 (s), 98.69 (s), 65.56 (d; J = 6.0 Hz), 15.88 (d; J = 7.2 Hz).

*Compound **10***: dark red, very viscous liquid, UV/vis (methanol) λ_{max} (nm) (log ϵ) 233 (4.81), 268 (4.68), 350 (4.73), 532 (3.96); IR (ATR) ν (cm^{-1}) 2978 (w), 2929 (w), 2782 (w), 2701 (w), 1711 (m), 1574 (s), 1505 (s), 1374 (s), 1344 (s), 1257 (m), 1201 (s), 1159 (m), 1098 (w), 1020 (s), 924 (s), 820 (m), 791 (m), 760 (m) 694 (m); LC/MS (ESI+) m/z (relative intensity) = 626.14 (100%), 627.16 (30.6%), 628.10 (14.1%), 629.15 (3.6%), 630.12 (0.8%); accurate mass (mean of 6 measurements ± standard deviation) m/z = 626.1311 ± 0.0010 [M + H]$^+$ (calcd 626.1313 for $C_{26}H_{34}N_3O_7P_2S_2$); ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm) 9.14 (NH; s; 1 H), 7.40/6.94 (m, m; 2 H, 2 H), 7.25/7.23 (m, m; 2 H, 2 H), 6.53 (NH_2; s; 2 H), 5.67 (s; 1 H), 5.49 (s; 1 H), 4.21 (dq; 8 H; J = 7.0/10.2 Hz), 1.31/1.30 (t; 12 H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm) 180.65 (s), 154.34/150.20/146.80 (s), 147.45, 135.66 (d; J = 1.8 Hz), 147.11/146.48 (d, d; J = 7.8 Hz), 125.26/121.79 (s, s), 121.60/121.31 (d, d; J = 4.8 Hz), 96.46/89.03 (s), 65.06/64.98 (d, d; J = 6.0 Hz), 15.77/15.74 (d, d; J = 7.2 Hz).

Incubation of Aminoparathion with Polyphenols and PPO. To a solution of 10−40 μmol of caffeic acid, catechol, pyrocatechol, quercetin, or naringenin, respectively, in 0.25 mL of acetone were added 50 μL of a methanolic solution of aminoparathion (45.3 g/L), 3.75 mL of water, and 1 mL of an aqueous PPO solution (1 g/L, 1460 U/mL). Under occasional shaking the mixture was incubated at 25 °C for 24 h. After addition of 1 mL of methanol the mixture was centrifuged (3000 min^{-1}), the supernatant analyzed for aminoparathion by HPLC, and the precipitate washed four times with about 1 mL of water and lyophilized.

RESULTS AND DISCUSSION

Photoreduction of parathion **1** was postulated as stepwise reduction of the nitro group (**Figure 1**) (*1*−*3*). Although **3** and **4** have not been found individually, their evidental formation was proven by the condensation products **5** and **6**, respectively. During irradiation of **1** in the presence of 2-propanol (1 g/L) as model for hydroxylated cutin acids, now **3** and **4** could be identified by means of HPLC, LC/MS, and comparison with synthesized standards. After 6 h of irradiation, **3** increased to 4 mol % of the initial parathion, while **4** reached 0.25 mol % only. Contrarily, after irradiation of **1** in cyclohexene (1 g/L) as model for olefinic cuticle constitutents, only **5** and **6** could be determined (data not shown).

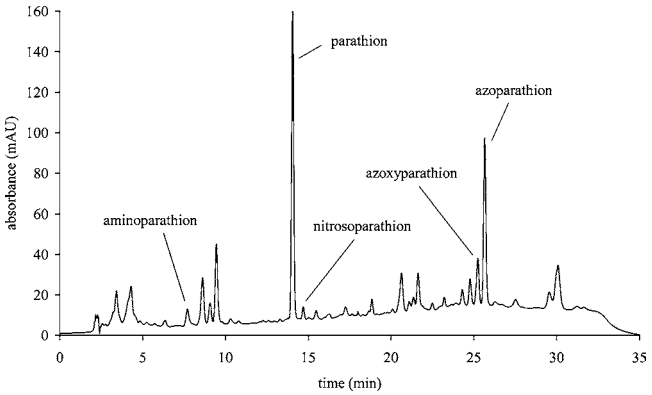

Figure 2. HPLC chromatogram of a fruit extract obtained after irradiation of an apple fortified with parathion (**1**, 134 mg/kg) and irradiated for 5 h (Suntest CPS+).

To examine the photochemical behavior of **1** on authentic plant material, an unrealistically high fortified apple (134 mg/kg) was irradiated, first. After 5 h of irradiation, the apple was extracted with methanol and a mixture of cyclohexane, diethyl ether, and butanone (50 + 45 + 5, v/v/v). The initial content of **1** was decreased to 111 mg/kg, and **2** and **4**−**6** could be detected in the extracts by means of HPLC (**Figure 2**). As on fruit surfaces **4** can only be photochemically formed by photoreduction of **3**, the absence of **3** in the extracts can be traced back to further reactions of **3** during the concentration steps performed before analysis. Second, irradiation experiments with tomatoes and apples were repeated at three lower fortification levels, which also will be reached in the field. For spiking, apples and tomatoes were dipped for 6 h in aqueous solutions of parathion as described by Wettach et al. (*7*), yielding initial levels of 0.70, 1.89, and 4.50 mg/kg for apples and 0.89, 1.28, and 3.79 mg/kg for tomatoes, respectively. After an irradiation time of 13 h more then 90% of the initial **1** levels could not be extracted from the fruits, anymore (**Figure 3**). Due to the fact that none of the known photoproducts were found by HPLC, the disappeared **1** had to become cuticle-bound, which was shown in former studies on enzymatically isolated cuticles by immunochemical methods (*7*).

Suzuki and Uchiyama described the reduction of **1** by spinach homogenate, yielding **2**−**4**, depending on an electron transport system (*9*−*11*). Although the authors identified the substances by specific reactions, they never isolated the metabolites nor specified constituents accountable for the reduction. Only the initial step, the reduction of **1** into **2**, was described as a cytochrome-dependent reduction. In model systems we could show that further reduction of the nitroso compound **2** is independent from complex plant electron transport systems. In the presence of ascorbic acid or thiols such as cysteine and glutathione, **2** was easily reduced yielding **3** and **4**, respectively. The total conversion ensued in less than 7 h (**Table 1**). Ascorbic acid, cysteine, and glutathione are common constituents of plants and reach, for instance, up to 83, 30, and 17 mg/100 g of fresh weight, respectively, in tomatoes (*23*). Therefore, it was shown for the first time that the formation of **3** and **4** in plants does not necessarily require light or enzymes but is simply a result of reducing agents present naturally, accompanied by increasing reactivity and further reactions with different plant constituents as polyphenols, for example.

Polyphenols such as catechol, quercetin, flavanols, or anthocyanidins, ubiquitously occur in plant materials. Catalyzed by polyphenol oxidase (PPO) polyphenols polymerize to mostly dark brown macromolecules with *ortho*-benzoquinoid structures

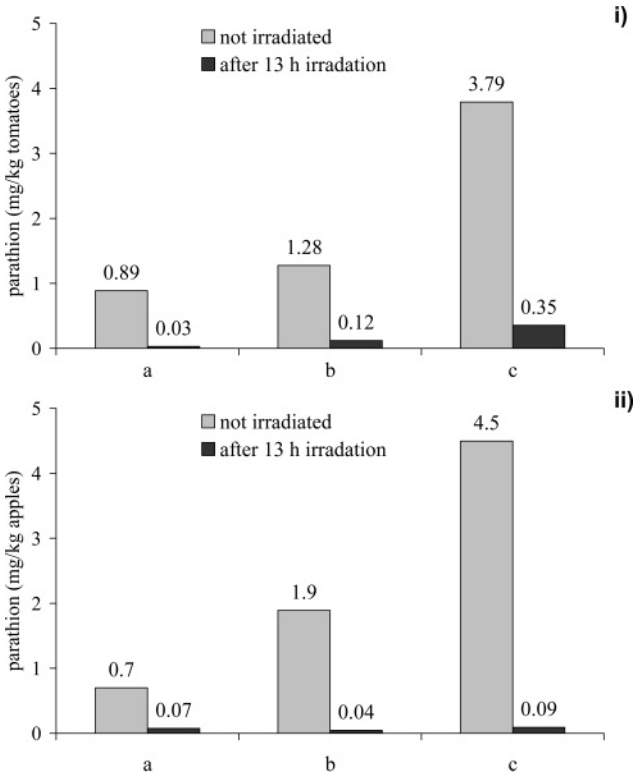

Figure 3. Extractable parathion (**1**) from tomatoes (i) and apples (ii) before and after irradiation (13 h, Suntest CPS+) at different fortification levels (a–c).

Table 1. Yields of (Hydroxylamino)parathion (**3**) and Aminoparathion (**4**) during Reduction of Nitrosoparathion (**2**) with Ascorbic Acid, Cysteine, and Glutathione (Data for Single Experiments)

react	reacn time (h)	**2** (mol %)	**3** (mol %)	**4** (mol %)
cysteine	0.5	4.4	nd[a]	69.0
	7.0	2.0	nd	71.6
	24.0	1.4	nd	72.3
glutathione	0.5	75.3	nd	2.1
	7.0	4.0	nd	37.0
	24.0	0.8	nd	60.6
ascorbic acid	0.5	1.5	88.9	nd
	7.0	1.4	88.7	nd
	24.0	2.3	27.6	nd

[a] nd: <0.5%.

as intermediates. The addition of nucleophiles, such as amines, to these α,β-unsaturated carbonyl intermediates is well-known by the formation of melanin, and therefore, the same reactivity should be expected for **4**. This hypothesis was first tested by oxidation of pyrocatechol with silver oxide in the presence of **4**. Already after a few minutes the color changed to red and **7** (**Figure 4**) could be isolated from the reaction mixture. To check, if **7** is also formed during oxidation of pyrocatechol by PPO, the PPO of potatoes was used exemplarily. Therefore, the polyphenols were removed from potatoes as described by Schaller (*24*), and pyrocatechol and **4** were incubated together with the obtained polyphenol-free potato powder (*25*). As expected, the color of the incubation turned to red and **7** could be identified by HPLC, demonstrating that the reaction of **4** with pyrocatechol is also catalyzed enzymatically.

Various authors described that not only phenolic compounds but also aromatic amines such as aniline serve as substrates for PPO, yielding *ortho*-benzoquinone imines (*26*, *27*). Therefore, the suitability of **4** as PPO substrate was tested by adding a

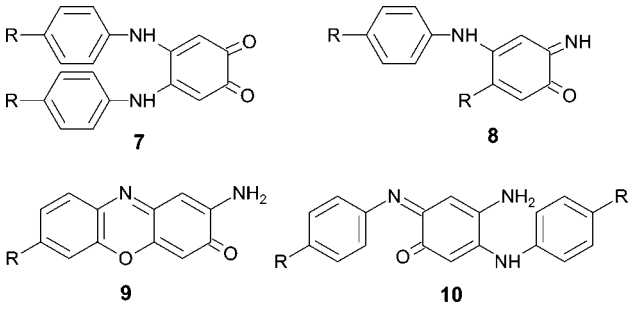

Figure 4. Structure formulas of products formed by PPO-catalyzed reactions of **4** (R = –OPS(OC$_2$H$_5$)$_2$).

Table 2. Recoveries of Pure Aminoparathion (**4**) and Polyphenol-Bound **4**, Determined as Triethyl Thiophosphate (TETP) after Treatment with Sodium Ethoxide

conjugate	recovery as TETP (%)
aminoparathion	53.1 (±2.0%)[a]
caffeic acid	57.0 (±2.5%)[a]
Catechol	34.6[b]
naringenin	34.6[b]
pyrocatechol	35.5[b]
quercetin	21.7[b]

[a] $n = 4$. [b] Data for single experiments.

methanolic solution of **4** to a phosphate buffered solution (pH 6.5) of PPO. After 24 h at ambient temperature, three red reaction products (**8**−**10**, **Figure 4**) could be isolated. The formation of **7**−**10** clearly shows that **4** coming into contact with plant material can be transformed into a couple of hitherto unknown products explaining the absence of **4** in residue analyses.

Besides pyrocatechol, the PPO-catalyzed reaction of **4** was also studied with different polyphenols common in fruits. Caffeic acid, pyrocatechol, quercetin, catechol, and naringenin were individually incubated with **4** in the presence of PPO in water. After 48 h, in all reaction mixtures a dark polymer precipitated, while **4** was not longer detectable, indicating that **4** was completely incorporated into the polyphenol polymers. The mixtures only differed in the colors of the supernatants. To prove the hypothesis of the incorporation, the isolated polymers were analyzed. While oxidation and other reactions only involve the amino group or the aromatic core, as was found for the products **7**−**10**, leaving the diethyl thiophosphate moiety unchanged, it should enable an approach for quantification of polyphenol bound **4**. Armbruster (*28*) described the determination of cuticle-bound residues of **1** by treating the cuticles with sodium ethoxide releasing triethyl thiophospate (TETP), which can be analyzed by means of GLC. According to this method we analyzed the isolated polymers and calculated the recoveries of **4** in terms of TETP. The relatively low recovery of 57% from **4** itself (**Table 2**) is in accordance with its lower phosphorylation activity as compared to parathion or paraoxon, respectively; *p*-aminophenol is the worse leaving group as compared to 4-nitrophenol, even in the presence of sodium ethoxide. However, from the isolated polyphenol polymers TETP was recovered in same order (22−58%). While the resulting polymer of caffeic acid offered the highest loading of **4**, the recovery from the quercetin polymer was least. But it has to be considered that incomplete (low grade) polymerization will yield in unknown soluble products also binding **4**, which, however, are lost by the focus on precepitated polymers. But the findings undoubtedly prove the hypothesis that **4** is readily and completely incorporated into polyphenol polymers formed by the action of PPO. To finally test authentic plant material, we spiked self-made apple juice with **4** (22 mg/L) and analyzed the juice after a storage time of 2 months, whereafter free **4** could not be determined anymore.

Conclusion. Disappearance of parathion from fruits and vegetables in the field will mainly be caused by photoreduction of the phenyl nitro group. However, due to the high reactivity of the parathion reduction products, they cannot be found as extractable residues. Besides the formerly known ene-type addition of nitrosoparathion to cuticular lipids, different PPO-catalyzed reactions of aminoparathion with or without polyphenols give rise for the formation of hitherto unknown parathion residue species which also cannot be analyzed by common methods of residue analysis. It is principally to be expected that the reaction types found for parathion are also transferable to other nitroaryl pesticides.

ABBREVATIONS USED

ATR, attenuated total reflection; PPO, polyphenol oxidase; TETP, triethyl thiophosphate.

ACKNOWLEDGMENT

We thank S. Reeb, Dr. J. Conrad, and Dr. B. Vogler (Institute of Chemistry, University of Hohenheim) for NMR measurements and PD Dr. M. O. Lederer (Institute of Food Chemistry, University of Hohenheim) for the GLC/MS measurements.

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Received for review June 27, 2005. Revised manuscript received September 6, 2005. Accepted September 8, 2005.

JF051520M