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"Untypical Aging Off-Flavor" in Wine: Synthesis of Potential Degradation Compounds of Indole-3-acetic Acid and Kynurenine and Their Evaluation as Precursors of 2-Aminoacetophenone

KATRIN HOENICKE,[†] OLE BORCHERT,[†] KAI GRÜNING,[†] AND THOMAS J. SIMAT*[,‡]

Institute of Biochemistry and Food Chemistry, University of Hamburg, Grindelallee 117, D 20146 Hamburg, Germany, and Institute of Food Chemistry, Technical University of Dresden, Bergstrasse 66, D 01062 Dresden, Germany

Kynurenine (**1**) and indole-3-acetic acid (**2**) are considered as potential precursors of 2-aminoacetophenone (**3**), which is regarded to be the aroma impact compound causing an "untypical aging off-flavor" (UTA) in *Vitis vinifera* wines. The mechanism of the formation of **3** was studied using model fermentation and model sulfuration media spiked with **1** or **2** as potential precursors. Possible degradation products such as kynurenamine (**4**) and kynurenic acid (**5**), or skatole (**6**), 2-oxoskatole (**7**), 2-formamidoacetophenone (**8**), 2-oxindole-3-acetic acid (**9**), and 3-(2-formylaminophenyl)-3-oxopropionic acid (**10**) were evaluated by HPLC−UV of the fermentation and sulfuration media and comparison with synthesized **7**, **8**, **9**, and **10**. The synthesis of the possible precursor 4-(2-aminophenyl)-2,4-dioxobutanoic acid (**11**), a proposed metabolite of **1** failed because a spontaneous cyclization yields **5** and *N*-oxo-kynurenic acid (**12**), but not **11**. It could be shown that the formation of **3** is triggered by an oxidative degradation of **2** after sulfuration with potassium bisulfite via the intermediates **10** and **8**. However, no formation of **3** occurred during sulfuration of a model wine spiked with **1** or during fermentation of a model must spiked with **1** or **2**.

KEYWORDS: 2-Aminoacetophenone; indole-3-acetic acid; kynurenine; synthesis; model fermentation; sulfuration; HPLC analysis; untypical aging off-flavor (UTA); *Vitis vinifera*

INTRODUCTION

2-Aminoacetophenone (**3**) is known as the aroma impact compound being responsible for an "untypical aging off-flavor" (UTA) in *Vitis vinifera* wines (*1*). The off-flavor can be formed in the bottle or in the wine cask within a few months after fermentation. UTA is described by aroma descriptors such as furniture polish, wet wool, mothbal, fusel alcohol, or acacia blossom combined with a loss of the typical bouquet of the grape variety. Depending on the wine, the off-flavor can be realized sensorically at 0.5−1.5 μg/L of **3** (*2*−*4*). Investigations on the time course of the formation of **3** during vinification revealed that its concentration is below the odor threshold in the berry, in the must, and in the wine directly after fermentation, and its significant formation occurred during storage of the sulfurized wines (*5*−*7*).

Compound **3** also causes off-flavors in products from milk such asstored dried lactic casein (*8*) or concentrated sterilized milk (*9*), as well as in products from corn (tortilla or taco chips (*10*)), and in beer (*11*). The biosynthesis of **3** is generally connected with the tryptophan metabolism (*12*, *13*). Several investigations have been done on the elucidation of the formation of **3** from tryptophan and its metabolites in human or animal organisms and by microorganisms. In wine especially, the tryptophan metabolites kynurenine (**1**) and indole-3-acetic acid (**2**) are discussed as potential precursors of **3** (**Figure 1A** and **B**). Studies by Kochen et al. (*14*) showed that the formation of **3** in untreated phenylketonuric patients is characterized by an enzymatic degradation of tryptophan via *N*-formylkynurenine and **1**. Kaseda et al. (*15*) discussed the synthesis of **3** from **1** in rat liver via an enzymatic decarboxylation yielding kynurenamine (**4**), which is nonenzymatically converted to **3**. Investigations of Yonaha et al. (*16*) and Shin et al. (*17*) on the metabolism of **1** in yeast showed that **1** is mainly metabolized by kynurenine transaminase to kynurenic acid (**5**). 4-(2-Aminophenyl)-2,4-dioxobutanoic acid (**11**) has been postulated as a precursor of **5** in this metabolic route (*18*). Considering the similarity of **11** to **3**, **11** can be supposed as a potential precursor of **3** (**Figure 1A**). The existence of a group of oxygenases in plants and animals which oxidizes the pyrrole ring of both **2** and skatole (**6**) yielding 2-formamidoacetophenone (**8**) and **3** was reported by Frydman et al. (*19*).

* To whom correspondence should be addressed. Tel: +49 351 463 31475. Fax: +49 351 463 34138. E-mail: thomas.simat@chemie.tu-dresden.de.
[†] University of Hamburg.
[‡] Technical University of Dresden.

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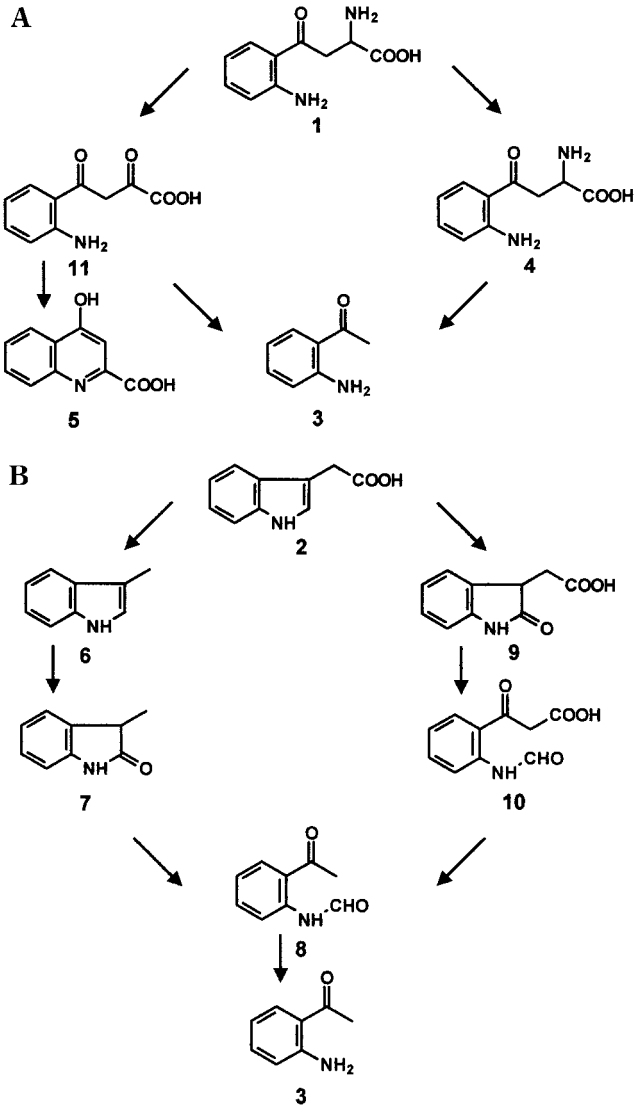

Figure 1. (A) Possible pathways of 2-aminoacetophenone formation from kynurenine (**1**, kynurenine; **3**, 2-aminoacetophenone; **4**, kynurenamine; **5**, kynurenic acid; **11**, 4-(2-aminophenyl)-2,4-dioxobutanoic acid). (B) Possible pathways of 2-aminoacetophenone formation from indole-3-acetic acid (**2**, indole-3-acetic acid; **3**, 2-aminoacetophenone; **6**, skatole; **7**, 2-oxoskatole; **8**, 2-formamidoacetophenone; **9**, 2-oxindole-3-acetic acid; **10**, 3-(2-formylaminophenyl)-3-oxopropionic acid).

In wine, besides an enzymatic formation of **3** by *Saccharomyces cerevisiae* during fermentation (*6*, *20*, *21*), a nonenzymatic mechanism was discussed. Christoph et al. (*22*) reported a formation of **3** by reaction of **2** with sulfite. They identified **6** and **8** as degradation products and postulated two possible degradation pathways: a decarboxylation occurs either prior to pyrrole ring oxidation yielding **6** and 2-oxoskatole (**7**) or after pyrrole ring oxidation yielding 2-oxindole-3-acetic acid (**9**) and 3-(2-formylamino)-phenyl-3-oxopropionic acid (**10**).

The aim of this study was to investigate possible pathways of the formation of **3** proceeding from the tryptophan metabolites **1** and **2** by analysis of their degradation compounds using different model fermentation and model sulfuration systems. We report the synthesis of potential degradation compounds and discuss the role of the identified substances as a precursor of **3**.

MATERIALS AND METHODS

Reagents. 2-Aminoacetophenone, indole-3-acetic acid, kynurenamine, kynurenic acid, kynurenine, and indole-3-aldehyde were obtained from Sigma (Deisenhofen, Germany), and skatole was from Merck (Darmstadt, Germany). *Saccharomyces cerevisiae* (Uvaferm CM 99/TH16) was provided by the Bayerische Landesanstalt für Weinbau und Gartenbau (Veitshöchheim, Germany).

Synthesis of Intermediate Compounds. *General.* The structures of the synthesized intermediate compounds were confirmed by ^{1}H- and ^{13}C-NMR, MS, and IR spectroscopy, as well as by melting-point (mp) determination and elementary analysis. The purities of the synthesized compounds were determined by HPLC−UV at 260 nm. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AMX 400 instrument (Karlsruhe, Germany) at 400 and 100.67 MHz, respectively, using deuterated solvents and tetramethylsilane as internal standard. Electron impact (EI) or fast atomic bombardment (FAB) mass spectra were obtained on a Varian 311 A instrument (Darmstadt, Germany) at 70 eV. IR spectra were measured using a Perkin-Elmer FT-IR 1720X spectrometer (Überlingen, Germany). Uncorrected mp determinations were made using an Olympus BH-2 polarization microscope (Hamburg, Germany).

Synthesis of N-oxo-Kynurenic Acid (12). Compound **12** was synthesized according to Makino et al. (*18*) in three steps via 4-(2-nitrophenyl)-2,4-dioxobutanoic acid ethyl ester and 4-(2-nitrophenyl)-2,4-dioxobutanoic acid. 4-(2-Nitrophenyl)-2,4-dioxobutanoic acid ethyl ester was prepared by dissolving 5 g of *o*-nitroacetophenone (30 mmol) and 4.4 g of diethyl oxalate (30 mmol) in 6 g of ethanol (99%). The mixture was added, under violent stirring and ice cooling, to a solution of 2 g of sodium ethylate (30 mmol) in 20 g of ethanol (99%) and then stirred for 1 h. After being left in the refrigerator overnight at −18 °C, the crystalline deposit was filtered, washed with diethyl ether, and subsequently dissolved in ice water. The solution was filtered and acidified with acetic acid. The acidified solution was centrifuged, and the crystalline deposit was dried and recrystallized from ethanol (99%) yielding 2.8 g of 4-(2-nitrophenyl)-2,4-dioxobutanoic acid ethyl ester (10 mmol, 34%). 4-(2-Nitrophenyl)-2,4-dioxobutanoic acid was then prepared by dissolving 2.4 g 4-(2-nitrophenyl)-2,4-dioxobutanoic acid ethyl ester (8 mmol) in 20 mL of 1 M sodium hydroxide. The solution was stirred for 30 min at room temperature, filtered, and acidified with hydrochloric acid (10%). This solution was centrifuged and the deposit was lyophilized yielding 1.12 g of 4-(2-nitrophenyl)-2,4-dioxobutanoic acid (4.8 mmol, 60%). **12** was obtained after treatment of 0.95 g of 4-(2-nitrophenyl)-2,4-dioxobutanoic acid (4 mmol) in 10 mL of ethanol (99%) with 100 μL of triethylamine and hydrogenation with a palladium-katalysator (250 mg, 10% on carbon) by stirring the solution for 1 h at −35 °C under hydrogen. The mixture was filtered, and the deposit was dissolved in ethanol (99%) and filtered again. Both filtrates were combined and the solvent was removed. The crystalline deposit was recrystallized from ethanol (99%). After the crystalline deposit was lyophilizied, 0.16 g of **12** was yielded (0.8 mmol, 20%).

4-(2-Nitrophenyl)-2,4-dioxobutanoic acid ethyl ester. $C_{12}H_{11}NO_6$. M = 265.2. Bright yellow crystals, mp 88−89 °C. Purity 96%. ^{1}H NMR: δ = 8.04 (1H, d, J = 7.6 Hz, H-10), 7.75 (1H, dd, J = 7.6 and 8.1 Hz, H-8), 7.68 (1H, dd, J = 7.6 and 8.1 Hz, H-9), 7.60 (1H, d, J = 7.6 Hz, H-7), 6.65 (1H, s, H-3), 4.38 (2H, q, H-11), 1.39 (3H, t, H-12) ppm. ^{13}C NMR: δ = 193.7 (C-1), 165.8 (C-2), 161.5 (C-4), 133.5 (C-9), 131.9 (C-8), 128.8 (C-10), 124.7 (C-7), 102.1 (C-3) 62.9 (C-11), 14.1 (C-12) ppm. MS (EI): m/z = 266 $[M + H]^+$ (0.3), 192 (28), 150 (100), 131 (10), 104 (14), 76 (26), 57 (12), 51 (18). MS (FAB): m/z = 266 $[M + H]^+$ (100). IR (KBr): 3102, 2998, 1729, 1639, 1573, 1524, 1475, 1418, 1368, 1348, 1313, 1256, 1112, 1054, 1017 cm^{-1}. Elementary analysis: C = 54.1% (54.3%), H = 4.1% (4.2%), N = 5.1% (5.3%).

4-(2-Nitrophenyl)-2,4-dioxobutanoic acid. $C_{10}H_7NO_6$. M = 237.2. Bright yellow crystals, mp 157−158 °C. Purity 99%. ^{1}H NMR: δ = 8.09 (1H, d, J = 7.6 Hz, H-10), 7.83 (1H, dd, J = 7.6 and 8.1 Hz, H-8), 7.74 (1H, dd, J = 7.6 and 8.1 Hz, H-9), 7.68 (1H, d, J = 7.6 Hz, H-7), 6.37 (1H, s, H-3) ppm. ^{13}C NMR: δ = 203.5 (C-1), 164.1 (C-2), 147.3 (C-4), 134.5 (C-9), 132.0 (C-8), 129.2 (C-10), 124.6 (C-7), 102.2 (C-3) ppm. MS (EI): m/z = 238 $[M + H]^+$ (0.3), 192 (27), 177 (3), 150 (100), 147 (54), 134 (18), 104 (18), 90 (15), 77 (20), 76 (47), 69 (12), 63 (14), 51 (32). MS (FAB): m/z = 238 $[M + H]^+$ (100). IR (KBr): 3084, 2894, 1734, 1628, 1599, 1538, 1444, 1410, 1377, 1274, 1243, 1131, 1035 cm^{-1}. Elementary analysis: C = 49.9% (50.6%), H = 2.9% (3.0%), N = 5.7% (5.9%).

12. $C_{12}H_7NO_4$. M = 205.2. Bright yellow crystals, mp 200−202 °C. Purity 95%. ^{1}H NMR: δ = 8.52 (1H, d, J = 8.7 Hz, H-8), 8.31 (1H, d, J = 7.1 Hz, H-5), 8.07 (1H, dd, J = 7.1 and 8.7 Hz, H-7), 7.86 (1H, dd, J = 7.1 and 7.1 Hz, H-6), 7.53 (1H, s, H-3) ppm. ^{13}C NMR: δ = 161.9 (C-9), 159.8 (C-4), 138.0 (C-2), 136.2 (C-8a), 133.5 (C-7), 129.0 (C-6), 123.5 (C-5), 122.8 (C-4a), 118.1 (C-8), 104.2 (C-3) ppm. MS (EI): m/z = 206 $[M + H]^+$ (0.1), 161 (57), 160 (26), 146 (11), 145 (100), 144 (9), 117 (60), 116 (48), 104 (28), 103 (10), 90 (36), 89 (44), 77 (33), 76 (36), 75 (10), 64 (16), 63 (28), 59 (12), 51 (16), 50 (26). MS (FAB): m/z = 206 $[M + H]^+$ (100). IR (KBr): 3105, 1696, 1603, 1532, 1450, 1381, 1301, 1275, 1241, 1202, 1159, 1115 cm^{-1}. Elementary analysis: C = 57.7% (58.5%), H = 3.6% (3.4%), N = 6.7% (6.8%).

*Synthesis of 2-Oxoskatole (**7**).* Compound **7** was prepared according to Savige and Fontana (*23*) by treating an ice-cooled solution of 5.0 g of **6** (38.1 mmol) in 25 mL of glacial acetic acid dropwise with a mixture of 4.5 mL of dimethyl sulfoxide and 12.5 mL of concentrated hydrochloric acid (37%). After the solution was stirred for 1 h at room temperature the pH was adjusted to 3 with ammonia (25%) under ice-cooling. Subsequently, 30 mL of ethyl acetate was added, and the organic layer was washed to neutral pH. The solvent was evaporated to dryness and the residue was purified by column chromatography on silica gel 60 (70−230 mesh). 1.5 g of **7** (10.2 mmol, 27%) was obtained using ethyl acetate as eluent. C_9H_9NO. M = 147.2. Bright yellow crystals, mp 121 °C. Purity 98%. ^{1}H NMR: δ = 7.22 (1H, d, J = 7.6 Hz, H-4), 7.21 (1H, dd, J = 7.1 and 7.6 Hz, H-6), 7.03 (1H, dd, J = 7.1 and 7.6 Hz, H-5), 6.90 (1H, d, J = 7.6 Hz, H-7), 3.44 (1H, q, J = 7.6 Hz, H-3), 1.50 (1H, d, J = 7.6 Hz, H-8α) ppm. ^{13}C NMR: δ = 181.2 (C-2), 141.1 (C-7a), 131.3 (C-3a), 127.9 (C-6), 123.8 (C-4), 122.4 (C-5), 109.7 (C-7), 41.0 (C-3), 15.2 (C-8) ppm. MS (EI): m/z = 147 $[M + H]^+$ (100), 132 (24), 128 (8), 119 (73), 104 (13), 47 (13). IR (KBr): 3197, 2972, 1714, 1678, 1621, 1473, 1402, 1378, 1337, 1232 cm^{-1}. Elementary analysis: C = 73.1% (73.5%), H = 6.0% (6.2%), N = 9.2% (9.5%).

*Synthesis of 2-Formamidoacetophenone (**8**).* According to Fürstner and Jumbam (*24*), a mixture of 1.0 g of **3** (7.4 mmol), 1.4 mL of acetic anhydride, and 2.2 mL of formic acid was stirred for 30 min under ice-cooling. After 2 h, 30 mL of diethyl ether and 15 mL of saturated sodium hydrogen carbonate solution were added. The aqueous layer was extracted twice with diethyl ether, and the organic phase was dried over sodium sulfate. After evaporation, the residue was recrystallized from ethanol yielding 0.95 g of **8** (5.8 mmol, 78%). $C_9H_9NO_2$. M = 163.2. Colorless crystals, mp 78−79 °C. Purity 99%. ^{1}H NMR: δ = 11.59 (1H, bs, −NH), 8.71 (1H, d, J = 7.6 Hz, H-3), 8.43 (1H, s, −CHO), 7.90 (1H, d, J = 7.9 Hz, H-6), 7.51 (1H, t, J = 7.5 and 7.9 Hz, H-5), 7.17 (1H, t, J = 7.5 and 7.6 Hz, H-4), 2.60 (3H, s, $-CH_3$). MS (EI): m/z = 163 $[M + H]^+$ (34), 148 (14), 135 (65), 120 (100), 92 (41), 65 (34). IR (KBr): 3253, 1685, 1647, 1604, 1579, 1516, 1454, 1406, 1390, 1361, 1309, 1254, 1211 cm^{-1}. Elementary analysis: C = 66.2% (66.3%), H = 5.6% (5.6%), N = 8.6% (8.6%).

*Synthesis of 2-Oxindole-3-acetic acid (**9**).* Compound **9** was synthesized according to Lawson and Witkop (*25*) by treating 5.0 g of **2** (28.5 mmol) in 250 mL of acetic acid (50%) with 9.2 g of *N*-bromsuccinimide (57 mmol) in 25 mL of glacial acetic acid. The mixture was stirred for 1 h at room temperature. A palladium-katalysator (500 mg, 10% on carbon) was added, and the solution was stirred for further 17 h under hydrogen, filtered, and the solvent was removed. The residue was poured into water, extracted with ethyl acetate, and dried with sodium sulfate. The solvent was removed, and the crystalline deposit was recrystallized from a mixture of toluol/diethyl ether (50:50, v:v) yielding 2.9 g of **9** (15.2 mmol, 52%). $C_{10}H_9NO_3$. M = 191.2. Bright yellow crystals, mp 142 °C. Purity 98%. ^{1}H NMR: δ = 7.21 (1H, d, J = 7.6 Hz, H-4), 7.15 (1H, dd, J = 7.6 and 7.6 Hz, H-6), 6.90 (1H, dd, J = 7.6 and 7.6 Hz, H-5), 6.80 (1H, d, J = 7.6 Hz, H-7), 3.62 (1H, dd, H-3), 2.89 (1H, dd, H-8β), 2.69 (1H, dd, J = 17.3 Hz, H-8α) ppm. ^{13}C NMR: δ = 184.3 (C-9), 178.3 (C-2), 133.1 (C-6), 129.3 (C-4), 127.0 (C-5), 114.9 (C-7), 47.6 (C-3), 40.1 (C-8) ppm. MS (EI): m/z = 191 $[M + H]^+$ (20), 145 (100), 117 (64), 99 (19), 90 (38), 78 (51), 69 (9), 63 (14), 56 (23), 51 (21). IR (KBr): 3250, 1735, 1684, 1621, 1487, 1472, 1408, 1341, 1266, 1214, 1155, 1105, 1020 cm^{-1}. Elementary analysis: C = 62.2% (62.8%), H = 4.8% (4.7%), N = 7.2% (7.3%).

Table 1. Detection Limits (μg/L) of **1**–**10** Using the Respective HPLC Conditions Described under HPLC Analysis (**1**, Kynurenine; **2**, Indole-3-acetic Acid; **3**, 2-Aminoacetophenone; **4**, Kynurenamine; **5**, Kynurenic Acid, **6**, Skatole; **7**, 2-Oxoskatole; **8**, 2-Formamidoacetophenone; **9**, 2-Oxindole-3-acetic Acid; **10**, 3-(2-Formylaminophenyl)-3-oxopropionic Acid)

	UV_{240nm}	UV_{260nm}
1	10	
2		73
3	5	48
4	12	
5	8	
6		47
7		47
8		26
9		103
10		104

*Synthesis of 3-(2-Formylaminophenyl)-3-Oxopropionic Acid (**10**).* **10** was prepared according to Schöpf et al. (*26*) by suspending 5.2 g of **2** (29.7 mmol) in 100 mL of tetrahydrofuran and 3 mL of methanol. The solution was aerated with oxygen (containing 2 vol-% ozone) for 3 h at −78 °C. After warming up to 0 °C, a palladium-katalysator (500 mg, 10% on carbon) was added. The solution was stirred for 2 h under hydrogen, filtered, and the solvent was removed. The residue was washed with ethyl acetate, and the precipitate was isolated and recrystallized twice from methanol yielding 2.0 g of **10** (9.7 mmol, 33%). $C_{10}H_9NO_4$. M = 207.2. Colorless crystals, mp 237 °C. Purity 98%. MS (FAB): m/z = 208 $[M + H]^+$ (15), 154 (100), 136 (73). A characterization by ^{1}H- and ^{13}C-NMR failed because of the instability of **10**. IR (KBr): 3340, 1734, 1673, 1584, 1519, 1453, 1407, 1326, 1302, 1205, 1151 cm^{-1}. Elementary analysis: C = 58.5% (58.0%), H = 4.3% (4.4%), N = 6.8% (6.8%).

Model Fermentation Medium. A must-like model solution consisting of glucose (95 g/L), fructose (95 g/L), ethanol (5 g/L), malic acid (3.5 g/L), tartaric acid (3 g/L), $MgSO_4 \cdot H_2O$ (1.65 g/L), K_2HPO_4 (1.5 g/L), $CaCl_2 \cdot H_2O$ (0.5 g/L), and diammoniumphosphate (1 g/L), adjusted to pH 3.5 with 1 M sodium hydroxide, was spiked with 100 mg/L **1** or **2**, respectively. The fermentations were carried out in 1-L flasks, and incubated on a shaker (100 U min^{-1}) at room temperature after inoculation with 20 g/hL *S. cerevisiae*. Samples were taken at different intervals during fermentation over a period of four weeks. For HPLC analysis samples were filtered through a 0.2 μm microfilter (Schleicher & Schuell, Dassel, Germany).

Sulfuration of Model Wines. A wine-like model solution consisting of ethanol (10 vol%), tartaric acid (5 g/L), and malic acid (5 g/L), adjusted to pH 3.3 with 1 M sodium hydroxide, was spiked with 200 mg/L of **1**, **2**, or other potential precursors and sulfurized with 100 mg/L potassium bisulfite. The solutions were stored at room temperature, or at 45 °C to simulate storage at room temperature for several months. Samples were taken in different intervals over a period of three weeks.

HPLC Analysis. *Determination of **1** and its Relevant Degradation Products **3**−**5** (HPLC−UV_{240nm}).* The HPLC−UV_{240nm} system consisted of a Merck 655A-12 liquid chromatograph, a Merck L-5000 LC controller, a Merck T-6300 column thermostat, and a LDC/Milton Roy SpectroMonitor D variable wavelength detector (Milton Roy, Hasselroth, Germany), set at 240 nm. Signals were acquired and calculated using ChromStar software (SCPA, Stuhr, Germany). Chromatographic separations were carried out on a Nucleosil 120-3 C_{18} column (250 mm × 4 mm) (CS, Langerwehe, Germany) equipped with a precolumn (20 mm × 4 mm) using a binary gradient (solvent A, 0.1% trifluoroacetic acid in bidistilled water; solvent B, methanol). The following gradient was used: 0 min, 5% B; 25 min, 40% B. The column was washed for 2 min with 40% B after each run and equilibrated for 8 min at the starting conditions. The flow rate was set to 0.8 mL/min, the injection volume was 20 μL, and the temperature of the column thermostat was 35 °C. Detection limits are given in **Table 1**.

*Determination of **2** and Relevant Degradation Products **3**, **6**−**10** (HPLC−UV$_{260nm}$).* The HPLC−UV$_{260nm}$ system consisted of a TSP AS 100 autosampler with integrated thermostat (Thermo Separation Products, Egelsbach, Germany), a Merck L 6200 A pump, and a Merck L 4250 UV−Vis detector, set at 260 nm. Signals were acquired and calculated using the ChromStar software. Chromatographic separations were carried out on a Nucleosil 120-3 C_{18} column (250 mm × 4 mm) equipped with a precolumn (20 mm × 4 mm) using a ternary gradient (solvent A, 0.1% trifluoroacetic acid in bidistilled water; solvent B, methanol; solvent C, acetonitrile). The following gradient was used: 0 min, 7% B, 11% C; 30 min, 7% B, 11% C; 40 min, 7% B, 50% C. The column was washed for 10 min with 7% B, 50% C after each run and equilibrated for 8 min at the starting conditions. The flow rate was set to 0.8 mL/min, the injection volume was 20 μL, and the temperature of the column thermostat was 31 °C. Detection limits are given in **Table 1**.

HPLC−UV/MS. The HPLC−UV/MS analysis was performed on a HP 1100 series HPLC system (Agilent, Waldbronn, Germany) equipped with a HP 1100 variable UV detector set at 260 nm, and a HP 1100 mass selective detector equipped with an electrospray ionization (ESI) source. MS parameters were the following: positive or negative mode, respectively; drying gas flow, 10.0 L/min; drying gas temperature, 350 °C; nebulizer pressure, 60 psig; capillary voltage, 3500 V; and fragmentation voltage, 30 V. Chromatographic separations were carried out on a Nucleosil column 120-3 C_{18} (250 mm × 4 mm) equipped with a precolumn (20 mm × 4 mm) using a binary gradient (solvent A, ammoniumformiate in bidistilled water, 5 mM, pH 3; solvent B, acetonitrile). The following gradient was used: 0 min, 5% B; 50 min, 50% B; 52 min, 5% B. The column was equilibrated for 8 min at the starting conditions. The flow rate was set to 1 mL/min, the injection volume was 50 μL, and the temperature of the column thermostat was 35 °C. System control, data acquisition, and processing were performed with the Agilent Technologies LC/MSD ChemStation, Rev. A.08.03.

Preparative HPLC. The preparative HPLC system consisted of a Merck NovaPrep 200 pump fitted with a 5-mL sample loop, and a Merck L-7400 UV detector, set at 260 nm. Separations were carried out on a Merck Lichrospher 100-7 C_{18} preparative column (250 mm × 25 mm). The mobile phase was water/acetonitrile (99:1). The flow rate was 4 mL/min.

RESULTS AND DISCUSSION

The desired compounds were obtained in sufficient amounts by using previously published methods of synthesis, which were varied and optimized in order to yield reference substances in a high purity (>95%). With these compounds it was possible to establish two different HPLC methods for the separation of **1** or **2** and their respective relevant degradation products giving high resolution of all substances of interest (**Figure 2A** and **B**).

The synthesis of **11** according to the method of Makino et al. (*18*) in three steps via 4-(2-nitrophenyl)-2,4-dioxobutanoic acid ethyl ester and 4-(2-nitrophenyl)-2,4-dioxobutanoic acid failed. During hydrogenation of 4-(2-nitrophenyl)-2,4-dioxobutanoic acid only **5** and **12** were obtained. By varying the hydrogenation parameters only the yield of **12** could be changed, but **11** was not obtained. These findings are in accordance with the results reported by Musajo et al. (*27*), indicating that there is a spontaneous cyclization before a complete hydrogenation (**Figure 3**). Compound **3** was not detectable in any of the synthesis batches, indicating that a formation of **3** from **1** via **11** is very improbable.

Evaluation of 1 as a Potential Precursor. *Fermentative Formation of **3** from **1** by S. cerevisiae.* To investigate a fermentative formation of **3** from **1** a model must solution spiked with **1** was fermented with *S. cerevisiae*, and the degradation of **1** was monitored by HPLC−UV$_{240nm}$. Only the formation of **5** could be detected during fermentation. After four weeks 35% of **1** was consumed by the yeast and 2 mol% was metabolized to **5**. A formation of **3** could not be detected (<0.008 mol%).

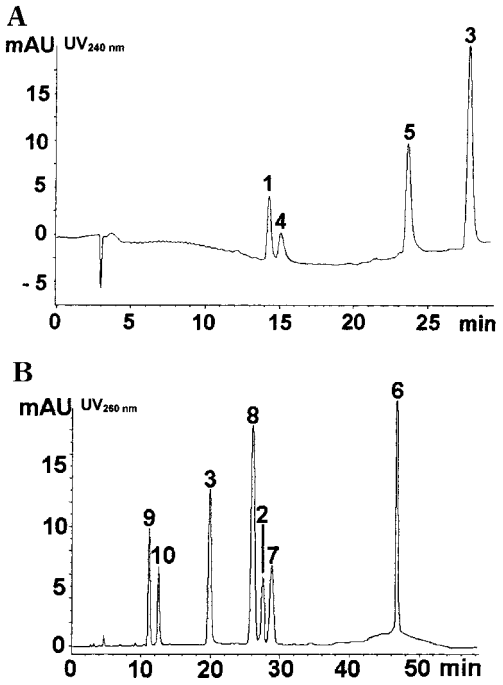

Figure 2. (A) HPLC–UV$_{240nm}$ chromatogram obtained from a standard mixture of kynurenine (**1**), 2-aminoacetophenone (**3**), kynurenamine (**4**), and kynurenic acid (**5**). (B) HPLC–UV$_{260nm}$ chromatogram obtained from a standard mixture of indole-3-acetic acid (**2**), 2-aminoacetophenone (**3**), skatole (**6**), 2-oxoskatole (**7**), 2-formamidoacetophenone (**8**), 2-oxindole-3-acetic acid (**9**), and 3-(2-formylaminophenyl)-3-oxopropionic acid (**10**).

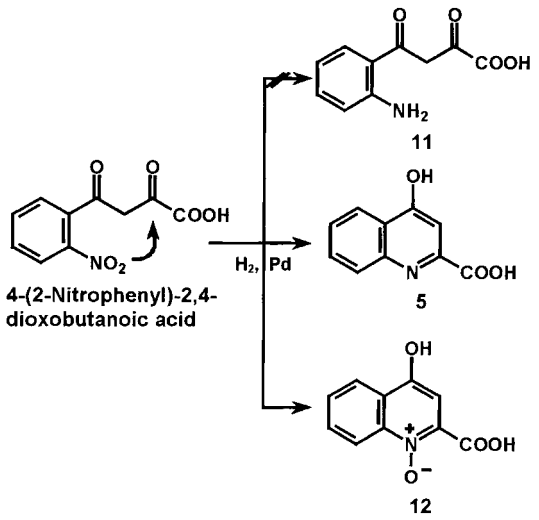

Figure 3. Formation of kynurenic acid (**5**) and *N*-oxo-kynurenic acid (**12**) by hydrogenation of 4-(2-nitrophenyl)-2,4-dioxobutanoic acid.

These findings confirm earlier studies by other authors on the metabolism of **1** in yeast, who describe its significant metabolization to **5** by kynurenine transaminase (*16*, *17*). However, Dollmann et al. (*21*) reported a possible formation of **3** by *S. cerevisiae*. After fermentation of a model must spiked with 80 mg/L of **1** they detected up to 55 μg/L of **3** in the wine, which complies with a conversion of 0.1 mol%. Our investigations excluded a relevant formation of **3** from **1** during fermentation. This finding is in accordance with results of Gessner et al. (*6*). The authors analyzed only 0.4 μg/L of **3** after fermentation of a must spiked with 25 mg/L of **1** and fermented with *S. cerevisiae*. This complies with a conversion of only 0.002 mol%.

*Nonfermentative Formation of **3** from **1** by Sulfuration.* A wine-like model solution was spiked with **1** and sulfurized, and

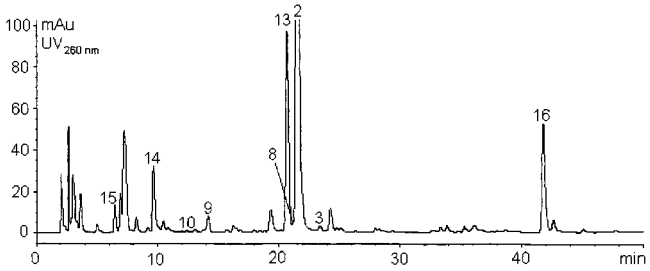

Figure 4. HPLC–UV/MS chromatogram obtained from a model wine solution spiked with **2** directly after sulfuration (**2**, indole-3-acetic acid; **3**, 2-aminoacetophenone; **8**, 2-formamidoacetophenone; **9**, 2-oxindole-3-acetic acid; **10**, 3-(2-formylaminophenyl)-3-oxopropionic acid; **13**, indole-3-aldehyde; **14**, (2-sulfoindole)-3-acetic acid; **15**, 2,3-dioxindole-3-acetic acid; **16**, 2-(3-indolylmethyl)-indole-3-acetic acid). Quantification of **8** was possible only by evaluation of its MS signal.

the degradation of **1** was monitored using HPLC−UV_{240nm}. Only the formation of **5** could be detected in this model system, too. After three weeks storage at 45 °C 20% of **1** had disappeared, but a formation of **3** was not detectable (<0.004 mol%). This finding is in accordance with results of Christoph et al. (*22*). They analyzed a conversion of <0.001 mol% by degradation of **1** added to a sulfurized ethanolic model solution. Because a formation of **3** from **1** could be observed neither by *S. cerevisiae* during fermentation nor by reaction with sulfite, **1** can be excluded as a likely precursor. Rather, our findings showed that a formation of **5** occurred both by fermentation and sulfuration of **1**.

Evaluation of 2 as a Potential Precursor. *Fermentative Formation of 3 from 2 by S. cerevisiae.* To investigate a fermentative formation of **3** from **2** a model must solution spiked with **2** was fermented with *S. cerevisiae*, and the degradation of **2** was monitored by HPLC−UV_{260nm}. In the model fermentation medium the formation of **9** could be detected. Furthermore, indole-3-aldehyde (**13**) was identified by comparison of its retention time and mass spectrum with the corresponding commercially available reference substance. After fermentation 46% of **2** was metabolized by the yeast or degraded by biotic and abiotic processes yielding 15 mol% of **9** and 3 mol% of **13**, but not **3** (<0.06 mol%).

Hühn et al. (*7*) detected a formation of about 45 μg/L of **3** directly after fermentation of a synthetic must medium with *S. cerevisiae* which was spiked with 100 mg/L of **2**. This complies with a conversion of 0.06 mol%. Gessner et al. (*6*) reported a conversion rate of 0.08 mol% after fermentation of a real must with *S. cerevisiae* to which 50 mg/L of **2** was added.

Regarding a natural pool of **2** in grape musts of 150 μg/L (*28*) a conversion of 0.08 mol% to **3** during fermentation will lead to only 0.09 μg/L of **3**, which is far below its odor threshold of about 0.5−1.5 μg/L. Therefore, a metabolism of **2** to **3** by *S. cerevisiae* during fermentation seems to have a relatively low importance concerning the formation of UTA.

Nonfermentative Formation of 3 from 2 by Sulfuration. A wine-like model solution spiked with **2** was sulfurized, and the degradation of **2** was monitored by HPLC−UV_{260nm} as well as by HPLC−UV/MS. Directly after sulfuration a large number of degradation products could be identified using HPLC−UV/MS (**Figure 4**). The oxidation products **9** and **13** were formed in this model system too. Moreover, traces of the degradation compounds **8** and **10** as well as **3** could be detected. The sulfuration product of **2** (2-sulfoindole)-3-acetic acid (**14**), 2,3-dioxindole-3-acetic acid (**15**), and 2-(3-indolylmethyl)-indole-3-acetic acid (**16**) were further identified by their respective mass

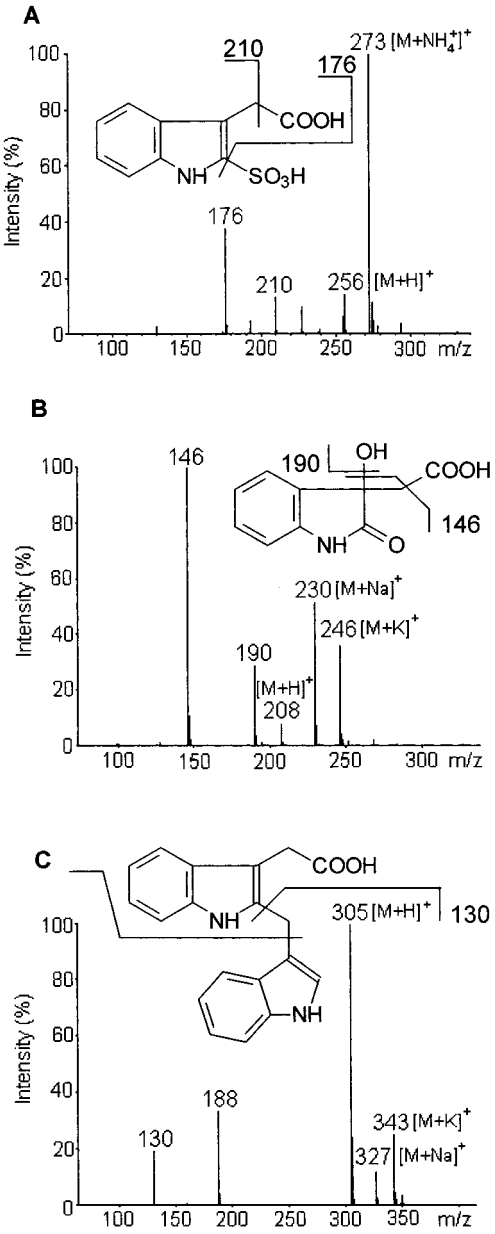

Figure 5. Mass spectra of the identified degradation products. A, (2-sulfoindole)-3-acetic acid (**14**); B, 2,3-dioxindole-3-acetic acid (**15**); C, 2-(3-indolylmethyl)-indole-3-acetic acid (**16**).

spectra (**Figure 5**). After three weeks of storage at room temperature, nearly 50% of **2** had disappeared while 7 mol% of **14**, 6 mol% of **13**, 6 mol% of **15**, 2 mol% of **9**, and 1 mol% of **3** were formed (quantities of the not-synthesized substances **14** and **15** were estimated according to the UV response of **2** or **9** as equivalents). **Figure 6** shows the amounts of **2** and its degradation products detected by HPLC−UV/MS directly after sulfuration, after 1 day, and after 3 weeks, respectively. Nearly 60 mol% of degraded **2** could be identified. Forty mol% of degraded **2 was** broken down into compounds not identified or not UV-active. The degradation of **2** and the formation of its degradation compounds occurred early after sulfuration, while in the later phase only a significant formation of **14** and **15** could be observed. Compounds **6** and **7** were not detected during the whole period of investigation (<0.06 mol%). In a blind test without addition of potassium bisulfite only **9** and **13** but no **3** (<0.03 mol%) were formed. According to other authors (29, *30*) the formation of **9** and **13** from **2** already occurs under the influence of atmospheric oxygen.

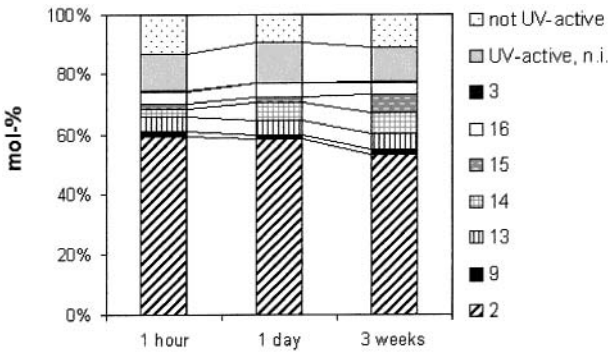

Figure 6. Amounts (mol%) of indole-3-acetic acid (**2**) and its degradation products detected directly after sulfuration, after 1 day, and after 3 weeks, respectively (**3**, 2-aminoacetophenone; **9**, 2-oxindole-3-acetic acid; **13**, indole-3-aldehyde; **14**, (2-sulfoindole)-3-acetic acid; **15**, 2,3-dioxindole-3-acetic acid; **16**, 2-(3-indolylmethyl)-indole-3-acetic acid); n.i., not identified. Amounts of **3**: 0.3 mol% (1 h); 0.4 mol% (1 day); 0.9 mol% (3 weeks).

The degradation compounds of **2** were also tested for their ability to form **3**. The potential precursors **14** and **15** were isolated by preparative HPLC. For this, 0.5 g of **2** was added to 500 mL of a wine-like model solution and sulfurized with 2.5 g of potassium bisulfite. The reaction mixture was kept at room temperature for 12 days. The solvent was concentrated to 50 mL by lyophilization. Purified fractions of **14** and **15** were obtained after preparative HPLC. One hundred mg/L of the identified degradation compounds **8**, **9**, **10**, **13**, **14**, or **15**, respectively, were added to a wine-like model solution and sulfurized, and their degradation was monitored by HPLC−UV/MS. Of those, only **8** and **10** could be elucidated as precursors of **3**. No formation of **3** occurred in the sulfurized model wine solutions spiked with **9**, **13**, **14**, or **15**. In the model wine spiked with **10** the formation of both **8** and **3** could be observed. These findings indicate that the formation of **3** from **2** occurs after a pyrrole ring cleavage of **2** yielding **10**, which is further decarboxylated giving **8** and subsequently **3.** However, an oxidation of the pyrrole ring yielding **9** or **15** did not lead to the formation of **3** under wine-like conditions. Therefore, the degradation pathway of **2** via **9** and **10** proposed by Christoph et al. (*22*) and shown in **Figure 1B** was not confirmed.

The oxidative degradation of **2** in the presence of sulfite and traces of oxidizing compounds was described by Horng and Yang (*31*). According to the authors superoxide, hydroxyl, and sulfite radicals, which were generated by the oxidation of sulfite to sulfate with residual oxygen, led to the formation of **14** and **15**. Since **14** and **15** were proven to not be precursors of **3** under wine-like conditions the reported mechanism is not likely to be relevant in the formation of **3**.

In a previous experiment (*32*) we proved that superoxide radicals are mandatory for the formation of **3**. On the contrary, **9** or **15** were formed by reaction of **2** with hydroxyl radicals or both hydroxyl and superoxide radicals, respectively. Taking this into account, as well as the results of the in vitro oxidations of the synthesized compounds, the mechanism shown in **Figure 7** can be assumed for the formation of **3** in wine from **2** after sulfuration. In accordance with other authors (*31*, *33*, *34*) the first step of the oxidative degradation of **2** is a one-electron oxidation at the 3-position of the indole ring in which both superoxide and hydroxyl radicals may function as a one-electron oxidant yielding the cation radical. By reaction of the radical cation with a superoxide radical 3-hydroperoxyindolenine-3-acetic acid can be formed (*31*, *33*). An intramolecular nucleophilic addition of the hydroperoxide to the indolenine double bond followed by a ring opening yields **10.** As was shown in

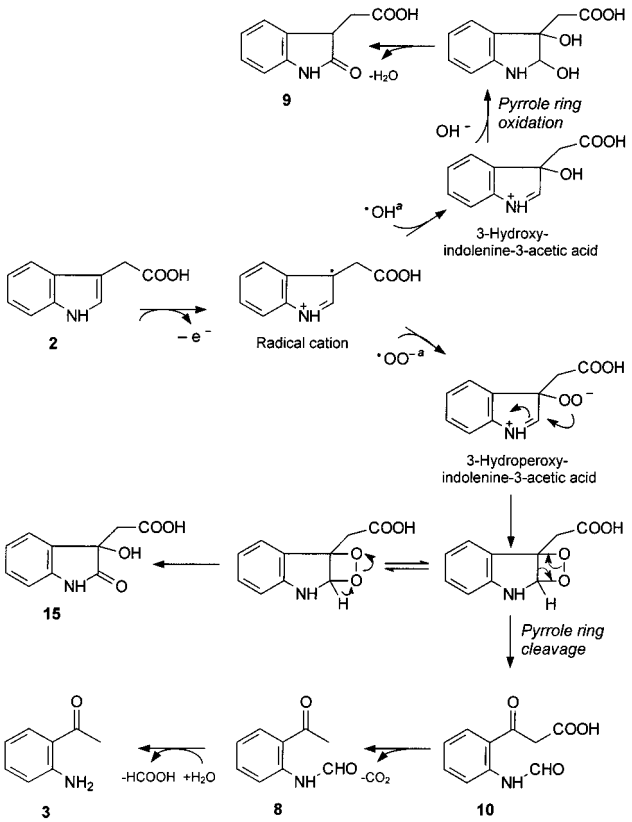

Figure 7. Proposed formation of 2-aminoacetophenone in wine triggered by an oxidative degradation of indole-3-acetic acid after sulfuration with potassium bisulfite (**2**, indole-3-acetic acid; **3**, 2-aminoacetophenone; **8**, 2-formamidoacetophenone; **9**, 2-oxindole-3-acetic acid; **10**, 3-(2-formylaminophenyl)-3-oxopropionic acid; **15**, 2,3-dioxindole-3-acetic acid). Superscipt a ([a]) designates a pathway investigated in ref *32*.

our experiments **10** is spontaneously decarboxylated giving **8** and subsequently **3.** However, we could not detect the formation of the postulated intermediates such as 3-hydroperoxyindolenine-3-acetic acid. Therefore, the mechanism presented in **Figure 7** is a plausible hypothesis.

In conclusion, in model fermentations no formation of **3** was detected from either **1** or **2**. Considering both precursors were spiked 1000 times the amount naturally occurring, a fermentative pathway from both precursors to **3** seems to be unlikely. On the other hand, the formation of **3** was detected in a model wine spiked with **2** after sulfuration, but not in a model wine spiked with **1**. The experiments with **2** as a possible precursor of **3** in wine-like model systems revealed an oxidative pathway from **2** via **10** and **8** to **3**. We conclude that a formation of **3** in wine is most likely to occur after sulfuration, which is indispensable for white wine making. However, these experiments do not rule out that other factors might also contribute to the formation of **3** in wine, e.g., the amounts of transition metals and antioxidants, the altered metabolism by the yeast due to the nutrient composition of the corresponding must, and other influences which are still unknown. Because **3** is an impact compound contributing to the off-flavor of UTA the described oxidative pathway from **2** to **3** might be more or less important for UTA formation in wine. A prevention of UTA by addition of ascorbic acid prior to sulfuration as reported by Gessner et al. (*35*) might give evidence for this hypothesis. Nevertheless, UTA description varies significantly from one winegrowing area to another, indicating that other aroma compounds besides **3** may play an important role which cannot be explained by the mechanisms described in this study.

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