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The first expedient entry to the human melanogen 2-*S*-cysteinyldopa exploiting the anomalous regioselectivity of 3,4-dihydroxycinnamic acid–thiol conjugation

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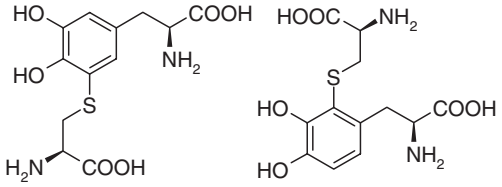
Abstract—The first convenient synthesis of 2-*S*-cysteinyl-3,4-dihydroxyphenylalanine (2-*S*-cysteinyldopa) in 30% overall yield is reported, which capitalizes on the anomalous regiochemistry of the oxidative coupling of 3,4-dihydroxycinnamic acid derivatives with thiol compounds, leading to 2-*S* rather than the usual 5-*S* conjugates.

The cysteinyldihydroxyphenylalanines (or cysteinyldopas), for example, **1**, **2**, are central melanocyte metabolites involved in the biosynthesis of pheomelanins, the characteristic pigments of red-haired, fair-complexioned individuals at high risk for melanoma and other skin cancers.[1] Besides being recruited for pheomelanin synthesis, these metabolites are partly excreted into body fluids and are among the best diagnostic markers of disseminated malignant melanoma.[2] Moreover, their metal chelating[3] and antioxidant properties[4] point to important, though still unclear, functional roles. Availability of cysteinyldopa isomers on a gram-scale is therefore central for addressing a number of chemical, biological and clinical issues relating, for example, to the structure and photoprotective/photosensitizing properties of pheomelanins, to the functional significance of circulating melanogens, and to their role in the etiopathogenesis of malignant melanoma.



1 **2**

Current synthetic methodologies involve oxidative conjugation of cysteine with 3,4-dihydroxyphenylalanine (dopa) or a derivative mediated by a range of oxidizing systems, including cerium ammonium nitrate (CAN) in sulfuric acid,[5] silver oxide in methanol,[6] Fe^{2+}-EDTA/H_2O_2[7] or tyrosinase[8] in neutral aqueous buffers. As a rule, 5-*S*-isomer **1**[9] is obtained as the major product (14–70% isolated yields), reflecting the dominant regiochemical course of the oxidative conjugation of 4-alkylcatechols with thiols,[10,11] whereas 2-*S* isomer **2** is formed in much lower amounts (<5% in our hands, despite slightly higher claims in the literature). To date, separation from the reaction mixtures provides the only means of obtaining small amounts of **2** for biological studies and/or analytical purposes. This, however, requires a lengthy and cumbersome procedure involving repeated ion-exchange column chromatography with HCl gradients.[5–8] In fact, the regiochemical issue in catechol–thiol conjugation has so far prevented a gram-scale preparation of **2**, despite the importance of this isomer in pheomelanin build-up.[12]

Our entry to this goal relied on the observation that caffeic acid and related 3,4-dihydroxyphenylpropenoic acid derivatives react with thiol compounds under oxidative conditions to give mainly 2-*S*-conjugates,[13–15] instead of the usual 5-*S*-conjugates that are formed with simple 4-alkylcatechols, such as dopa, dopamine, and 4-methylcatechol.[1,10,11] This anomalous regiochemistry has been rationalized in terms of the effects of the conjugated propenoate chain enhancing the coefficient of the LUMO of

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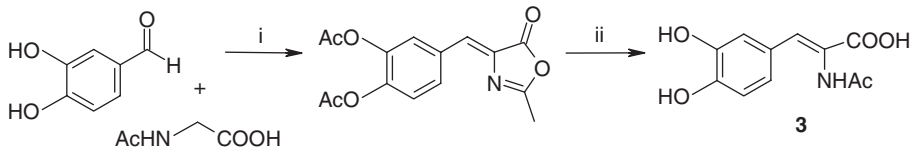
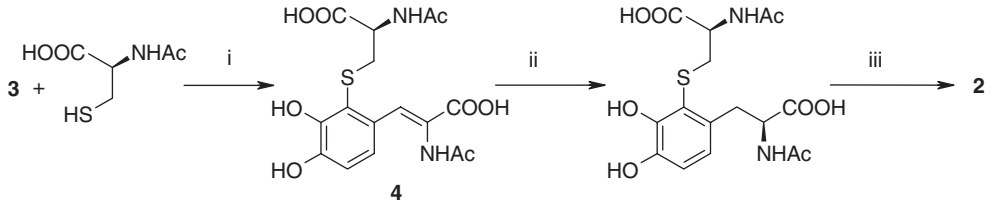


Scheme 1. Synthesis of **3** from 3,4-dihydroxybenzaldehyde and *N*-acetylglycine. Reagents and conditions: (i) Acetic anhydride, 120 °C, 5 h; (ii) 0.2 M HCl, under reflux, 1 h.



Scheme 2. Oxidative conjugation of **3** with *N*-acetylcysteine and stereoselective reduction of the adduct. Reagents and conditions: (i) Tyrosinase (50 U/mL), rt, 2 h; (ii) H_2, 3 mol % chiral catalyst, rt, 50 atm, 72 h; (iii) 3 M HCl reflux, 5 h.

the corresponding *o*-quinones at the 2-position of the quinone ring.[15] Capitalizing on this observation, a synthetic procedure was devised, which revolves around an amino-substituted 3,4-dihydroxyphenylpropenoic acid, which would expectedly form a 2-*S*-adduct by oxidative conjugation with L-cysteine. Stereoselective reduction of the propenoate moiety would then afford the desired product **2** with both amino acids in the natural L-configurations.

Accordingly, 2-acetylamino-3-(3,4-dihydroxyphenyl)-propenoic acid (**3**) was prepared by a Knoevenagel-type condensation of 3,4-dihydroxybenzaldehyde with *N*-acetylglycine in acetic anhydride at 120 °C followed by hydrolysis of the ethyl acetate extracts containing the oxazolone intermediate (Scheme 1).[16] After the removal of some unreacted starting material by ethyl ether extraction, product **3** (>95% purity) was isolated in 60% yield.[17]

Several oxidants were then screened to install the cysteinyl moiety onto the desired position of **3**. To this aim, *N*-acetylcysteine was preferably used in the place of cysteine to prevent intramolecular cyclization of the amino group on the quinone produced by oxidation of **3**. Under a variety of conditions, however, di- and tricysteinyl adducts were invariably obtained as main products because of the increased ease to oxidation of the initial *S*-conjugate with respect to the starting material. This difficulty was overcome by using commercially available mushroom tyrosinase as the oxidant. Thus, in line with our expectations, enzyme-promoted oxidation of **3** in the presence of 2 M equiv of *N*-acetylcysteine in aqueous buffer at pH 7.4[18] resulted in a complete consumption of the starting material after 2 h with the formation of the desired (*Z*)-2-*S*-cysteinyl-3-(3,4-dihydroxyphenyl)-2-acetylaminopropenoic acid (**4**),[19] isolated in 55% yields by preparative HPLC (Scheme 2).

Asymmetric hydrogenation of the *Z*-double bond in **4** was achieved in the presence of a rhodium chiral biphosphine catalyst[20] in a Parr apparatus.[21] Purity of the starting material with respect to residual cysteine contamination proved critical because of catalyst poisoning, and hence a chromatographic purification prior to hydrogenation proved necessary. Under 50 atm hydrogen pressure, with a 3 mol % catalyst loading and methanol as the solvent, the reaction was complete within 72 h (Scheme 2). Acid hydrolysis of the hydrogenation mixture under reflux eventually afforded **2** in 90% yield with a diastereoisomeric excess of 90% by HPLC analysis.[21] Product identity was secured by comparison with an authentic sample,[6,8] while purity was checked by NMR analysis.[21]

In conclusion, we have developed the first direct synthesis of the human melanogen **2** by a convenient three-step synthesis in overall 30% yield. This is by far the highest yield for this biologically and clinically relevant amino acid, which has so far been obtained only as a minor side product in the synthesis of **1**. The procedure is simple, efficient, scaleable and is expected to open up the doorway to novel insights into pheomelanin structure and functions. Moreover, the approach proposed in this study may be of value as a general access route to synthetic targets featuring 'anomalous' 2-*S* thiol–catechol conjugate substructures.

Acknowledgments

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17. The reported procedure[16] was followed with slight modifications. 3,4-Dihydroxybenzaldehyde (1.0 g, 7.3 mmol), *N*-acetylglycine (1 g, 8.5 mmol), and sodium acetate (2.3 g, 28 mmol) were heated at 120 °C in acetic anhydride (7.8 mL) under stirring. After 5 h the reaction mixture was diluted with 0.1 M phosphate buffer (pH 7.4) and extracted with ethyl acetate. The residue obtained from the organic layers was taken up in 0.2 M HCl (74 mL) and refluxed for 1 h. After ethyl ether washing the mixture was taken to dryness to afford **3**[16] (1.0 g, 60% yield) as yellowish powder (>95% pure by HPLC and ^{1}H NMR analyses). ESI+/MS: *m*/*z* 238 $[M+H]^+$, 260 $[M+Na]^+$.
18. A solution of **3** (500 mg, 2.1 mmol) in 0.01 M phosphate buffer (pH 7.4) (1.15 L) was sequentially treated with *N*-acetyl-L-cysteine (688 mg, 4.2 mmol) and mushroom tyrosinase (50 U/mL) and the mixture was taken under vigorous stirring at rt. After 2 h a complete consumption of the starting product was obtained (HPLC evidence, 0.1 M formic acid/methanol 88:12) and the mixture was acidified to pH 3 and fractionated by preparative HPLC (10 μm particle size 250 × 22 mm Econosil C18, eluant as above, 30 mL/min) to afford **4** (460 mg, 55% yield). Purity >95% as determined by ^{1}H NMR analysis.
19. (*Z*)-2-Acetylamino-3-[2-(2-acetylamino-2-carboxyethylthio)-3,4-dihydroxyphenyl]propenoic acid (**4**). HR ESI+/MS: found *m*/*z* 399.1049 ($[M+H]^+$), calcd for $C_{16}H_{19}N_2O_{10}$ *m*/*z* 399.1040; UV λ_{max} (CH_3OH) 257, 314; $[\alpha]_D$ +44 (*c* 0.25, CH_3OH); ^{1}H NMR (DMSO-d_6) δ (ppm) 1.82 (3H, s, $COCH_{3cys}$), 1.87 (3H, s, $COCH_3$), 2.85 (1H, dd, $J = 13.2$, 10.0 Hz, $-SCH_2$), 3.32 (1H, dd, $J = 13.2$, 4.4 Hz, $-SCH_2$), 3.98 (1H, m, $-CH_2CH$), 6.80 (1H, d, $J = 8.4$ Hz, H-6′), 6.96 (1H, d, $J = 8.4$ Hz, H-5′), 7.63 (1H, s, H-3) 8.16 (1H, d, $J = 8.0$ Hz, $NHCOCH_{3cys}$), 9.11 (1H, s, $NHCOCH_3$); ^{13}C NMR (DMSO-d_6) δ (ppm) 22.5 ($COCH_{3cys}$), 22.6 ($COCH_3$), 35.2 ($-SCH_2$), 52.2 ($CHCH_2$), 115.6 (C-5′), 120.0 (C-2′), 120.4 (C-6′), 126.0 (C-2), 128.7 (C-1′), 131.5 (C-3), 146.3 (C-4′), 147.2 (C-3′), 166.8 (C-1), 169.5 ($-NHCO$), 169.9 ($-NHCO_{cys}$), 172.6 ($COOH_{cys}$). Proton and carbon resonance assignment follows from 2D NMR experiments.
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21. To a 100 mL hydrogenation bomb were added a solution of **4** (800 mg) in methanol (30 mL) and cyclooctadiene-1,5[(*R*,*R*)1,2-ethanediylbis(*o*-methoxyphenyl)phenylphosphine]rhodium tetrafluoroborate (44 mg, Acros Organics). The solution was purged by filling and evacuating with N_2 and finally with H_2, and then allowed to stand under stirring at 50 atm for 72 h. After the removal of the solvent the residue was taken up in 3 M HCl (50 mL) and taken under reflux for 5 h under an argon atmosphere. Removal of the catalyst by filtration and evaporation of the acid gave a pale yellow oil, which was dissolved in ethanol and added to ethyl acetate to afford **2** as a colorless powder.[6,8] Diastereoisomeric excess was determined by HPLC analysis using a Synergi Hydro-RP 80A column (250 × 4.6 mm, 4 μm) using 0.1% trifluoroacetic acid/methanol 99:1 (0.7 mL/min) as the eluant, detection wavelength 280 nm. Retention times of **2** and its diastereoisomer under these conditions were 9.9 and 10.8 min, respectively. ^{1}H NMR (D_2O): δ (ppm) 3.18 (1H, dd, $J = 14.4$, 9.2 Hz, $-CHCH_2$), 3.31 (1H, dd, $J = 14.8$, 4.4 Hz, CH_{2cys}), 3.45 (1H, dd, $J = 14.8$, 7.2 Hz, CH_{2cys}), 3.66 (1H, dd, $J = 14.4$, 6.0 Hz, $-CHCH_2$), 4.12 (1H, dd, $J = 7.2$, 4.4 Hz, CH_{cys}), 4.23 (1H, dd, $J = 9.2$, 6.0 Hz, CH), 6.84 (1H, d, $J = 8.0$ Hz, H-6), 6.96 (1H, d, $J = 8.0$ Hz, H-5); ^{13}C NMR (D_2O): δ 36.1 ($-CHCH_2$), 36.4 (CH_{2cys}), 54.1 (CH_{cys}), 55.9 ($-CHCH_2$), 118.8 (C-5), 119.7 (C-2), 124.3 (C-6), 132.0 (C-1), 145.6 (C-4), 148.6 (C-3), 172.0 ($COOH_{cys}$), 173.3 (COOH). Proton and carbon resonance assignment follows from 2D NMR experiments.