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2,5-Bis-(2-hydroxybenzoylamino)pentanoic Acid, a Salicylic Acid-Metabolite Isolated from Chicken: Characterization and Independent Synthesis

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Abstract—From excreta of chickens that had been treated with sodium salicylate, a new compound was detected and identified as a double conjugated ornithine metabolite. The structural assignment of this metabolite was further confirmed by an independent efficient 3-step synthesis from ornithine.

Knowledge of xenobiotic metabolism in birds is, in contrast to mammals, very limited, especially with regard to drugs containing a carboxylic acid group, such as salicylates. In the body, a drug can be metabolized in two phases. In the first phase (phase I), the reactions occur which can be classified as oxydations, reductions and hydrolyses. The products of the first phase may then proceed to the second phase (phase II), which are conjugation reactions. A wide range of carboxylic acids compounds (drugs, herbicides, and pesticides) can conjugate to amino acids in living species. The fate of the simplest aromatic carboxylic acid, benzoic acid, has been studied extensively as a model compound for biotransformation reactions in animals, and it has been demonstrated to undergo conjugation with sugars (glucuronic acid in vertebrates, glucose in insects) or amino acids.[1] The nature of the amino acid varies markedly according to the animal species and the chemical structure of the carboxylic acid. In general, the amino acids participating in conjugation reactions are glycine, glutathione, taurine, and ornithine. Glycine and glutathione are most utilized by a wide range of species, including humans, for conjugation of carboxylic acids. Taurine conjugation is relatively widespread in most animal species, but is restricted to arylacetic and cholic acid derivatives.

Ornithine conjugation occurs only in some birds and reptiles, but it has not been reported in mammalian species.[2] Williams[3] and Seymour[4] reported that in birds belonging to the order of Galliformes (chicken, turkey, quail) and Anseriformes (duck, goose), the major metabolite of benzoic acid is ornithurate (α,δ-dibenzoylornithine), whereas in most mammals and in some bird species belonging to the order of Columbiformes (pigeon, woodpigeon, dove) conjugation with benzoylglycine with the formation of hippurate is present.

Salicylic acid (SA), usually administered as its precursor acetylsalicylic acid, is widely used for its analgesic, antipyretic and antiphlogistic activities. Acetylsalicylic acid is rapidly hydrolyzed to SA after absorption has

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been taken place. In humans, SA is metabolized to various metabolites. The main important metabolites are salicyluric acid (SU), also called the glycine conjugate, salicylic acid phenolic glucuronide (SAPG) and salicylic acid acyl glucuronide (SAAG). SU is also further conjugated with glucuronic acid to form salicyluric acid phenolic glucuronide (SUPG).[5]

The use of SA (a water soluble form of acetylsalicylic acid) and non-steroidal anti-inflammatory drugs (NSAID's) in general in bird medicine is still controversial. Economically important features such as growth and egg production seem not to improve by the use of NSAID's. Therefore, up till today, the availability of commercial formulations for use in poultry industry in most European countries is very limited. Nevertheless, valid indications for the use of an NSAID in birds may exist and treatment of certain pathological processes with NSAID's can be beneficial.[6] The pharmacokinetics of sodium salicylate in several bird species have been described by Baert and De Backer.[7] Hereby, a marked species difference in pharmacokinetic parameters was found between chicken and pigeon. A half-life of sodium salicylate in pigeons could be calculated which was three times longer than in chickens. According to these authors this large difference in elimination half-life would be mainly provided by a substantial difference in drug metabolism, as was already seen for benzoic acid.

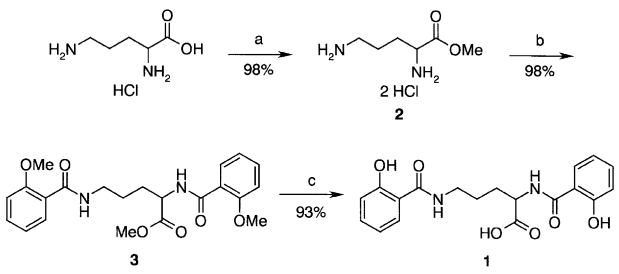
Metabolite Isolation

From excreta samples of chickens that had been treated with sodium salicylate, salicylic acid and its metabolites were isolated by a liquid-liquid back-extraction with diethyl ether as extraction solvent, followed by a solid-phase extraction procedure using a SAX column (strong anion exchange, Isolute, Sopachem, Brussels, Belgium). Chromatographic separations were achieved on a Nucleosil C_{18} column (100×3.0 mm i.d., Chrompack, Varian, Middelburg, The Netherlands), using a gradient elution with 1% acetic acid in water and methanol. The flow rate was 0.2 mL/min. A Quattro Ultima triple-quadrupole instrument (Micromass), equipped with an ESI Z spray™ source, which was operated in the negative ion MS/MS mode, was used as detector.

Synthesis

Since standard acylation methods using acetylsalicyloyl chloride yielded complex reaction mixtures, we opted for a synthetic sequence as depicted in Scheme 1. Treatment of ornithine.HCl with thionylchloride in methanol led to methyl 2,5-diaminopentanoate (**2**).[8] Acylation of **2** with *o*-anisoylchloride furnished amide **3**, which was subsequently treated with BBr_3[9] to give the desired product **1** in an overall yield of 89%.

The structure of the desired product and the intermediates was assigned by ^1H NMR and ^{13}C NMR. Mass spectra were acquired on a quadrupole/orthogonal-



Scheme 1.[10] Synthetic pathways of the SA-conjugate **1**. Reagents and conditions: (a) $SOCl_2$, MeOH, reflux; (b) Et_3N, CH_2Cl_2, *o*-anisoylchloride, −15 °C; (c) BBr_3, CH_2Cl_2, rt.

acceleration time-of-flight (Q/oaTOF) tandem mass spectrometer (qTof 2, Micromass, Manchester, UK) equipped with a standard electrospray ionization (ESI) interface operated in the positive ionisation mode. Samples were infused in a propan-2-ol:water (1:1) mixture at 3 μL/min. Fragment ion spectra were acquired with a collision energy of 30 eV. Accurate mass measurements were performed using the lock mass of the protonated peptide YGGFL ($m/z = 556.2771$), which was added to the sample (10 pmol/μL). For accurate mass measurements of the fragments, the precursor ion peak was used as internal mass reference.

Results

From the aforementioned biological excreta, a compound was detected with negative ionization tandem mass spectrometry (LC-MS/MS) that had an m/z value of 371.2.

HRMS in the positive mode (Fig. 1) suggested that this metabolite has an elemental formula of $C_{19}H_{20}N_2O_6$ (calculated m/z 373.1399 for M + H), as this was the sole feasible composition for the measured value of M + H of 373.1407 ($C_{0\text{-}30}H_{0\text{-}40}N_{0\text{-}5}O_{0\text{-}10}$) with a tolerance of 5 ppm. The ready loss of a neutral 120 Da fragment from the pseudo-molecular ion suggests cleavage of a salicyloyl moiety. Based on these spectroscopic data, the substance was proposed to be 2,5-bis-(2-hydroxybenzoylamino)-pentanoic acid. All fragment ions (positive ion mode)

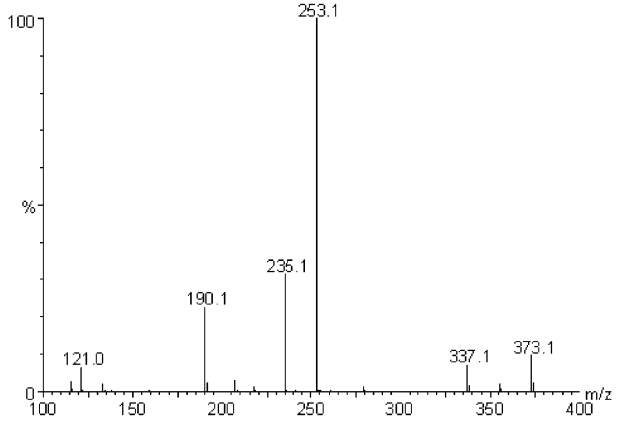


Figure 1. Mass spectrum (positive ion mode) of the salicyluric acid metabolite **1**.

Table 1. Assignment of all fragment ions (positive ion mode) in the mass spectrum of **1**

Ion (ppm)	Relative abundance (%)	*m/z* Measured	*m/z* Calculated	Elemental composition	Error
M + H–H_2O	1.9	355.1288	355.1294	$C_{19}H_{19}N_2O_5$	−1.7
M + H–2H_2O	7.0	337.1180	337.1189	$C_{19}H_{17}N_2O_4$	−2.9
M + H–salicyloyl + H	100	253.1178	253.1188	$C_{12}H_{17}N_2O_4$	−3.9
M + H–salicyloyl–H_2O + H	31.3	235.1068	235.1083	$C_{12}H_{15}N_2O_3$	−6.4
M + H–HCOOH–salicylamide	22.5	190.0858	190.0868	$C_{11}H_{12}NO_2$	−5.2
M + H–2 salicyloyl + 2H	2.0	133.0985	133.0977	$C_5H_{13}N_2O_2$	6.0
Salicyloyl	6.5	121.0310	121.0289	$C_7H_5O_2$	17
M + H–2 salicyloyl + 2H	2.8	115.0897	115.0872	$C_5H_{11}N_2O$	23

present in the mass spectrum can be assigned (Table 1). The structure may arise from double conjugation of salicylic acid to ornithine.

Structure **1** was confirmed by comparison of relevant spectroscopic data with those of the synthetic compound. The synthesized ornithine conjugate will further serve as a valuable tool (analytical standard) to investigate the excretion of salicylic acid and its metabolites.

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10. All new compounds were characterized by spectroscopic means. **3**: white solid; TLC $R_f = 0.37$ (ethyl acetate/hexane = 50:50); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.60 (2H, m, CH_2–CH), 1.87 (2H, m, CH_2–CH_2–CH_2), 3.29 (2H, dt, app dd, $J = 6.45$ and 12.61, NH-CH_2), 3.67 (3H, s, $COCH_3$), 3.84 (3H, s, OCH_3), 3.90 (3H, s, OCH_3), 4.53 (1H, ddd, $J = 5.28$, 7.92 and 12.61, C*H*-NH), 6.96–7.18 (4H, m, arom.), 7.40–7.52 (2H, m, arom.), 7.67 (1H, dd, $J = 2.05$ and 7.91, arom.), 7.76 (1H, dd, $J = 1.76$ and 7.62), 8.16 (1H, t, $J = 5.57$, *NH*-CH_2), 8.47 (1H, d, $J = 7.33$, CH–*NH*–CO); ^{13}C NMR (75 MHz, DMSO-d_6) δ 25.67, 28.73, 38.68, 52.10, 52.38, 55.88, 56.20, 112.05, 112.39, 120.52, 120.75, 122.30, 123.87, 130.26, 130.66, 131.98, 132.78, 156.93, 157.33, 164.94, 165.25, 172.56; Exact mass (ESI-MS) for $C_{22}H_{27}N_2O_6$ $[M + H]^+$: found 415.1861 calculated 415.1868. **1**: white solid; TLC $R_f = 0.24$ (ethyl acetate/hexane/formic acid = 80:20:1); ^{1}H NMR (300 MHz, DMSO-*d6*) δ 1.63 (2H, m, *CH*$_2$–CH), 1.88 (2H, m, CH_2–*CH*$_2$–CH_2), 3.34 (2H, dt, app dd, $J = 6.81$ and 12.81, NH–*CH*$_2$), 4.49 (1H, ddd, $J = 5.18$, 8.45 and 12.54, C*H*-NH), 6.82-6.95 (4H, m, arom.), 7.33–7.44 (2H, m, arom.), 7.82 (1H, dd, $J = 1.64$ and 8.45, arom.), 7.95 (1H, dd, $J = 1.64$ and 8.45, arom.), 8.80 (1H, t, $J = 5.72$, N*H*-CH_2), 8.93 (1H, d, $J = 7.63$, CH–*NH*–CO), 12.14 (1H, br s, OH), 12.65 (1H, br s, OH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 25.83, 28.46, 38.69, 52.28, 115.25, 115.72, 117.43, 117.56, 118.62, 118.93, 127.69, 128.78, 133.81, 133.95, 159.50, 160.41, 168.48, 169.29, 173.34; Exact mass (ESI-MS) for $C_{19}H_{21}N_2O_6$ $[M + H]^+$: found 373.1407 calculated 373.1399.