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# Synthesis of catecholamine conjugates with nitrogen-centered bionucleophiles

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ABSTRACT

The enzymatic (tyrosinase) and chemical ($NaIO_4$, $Ag_2O$ or Frémys's salt) oxidation of biologically relevant catecholamines, such as dopamine (DA), *N*-acetyldopamine (NADA) and the Ecstasy metabolites (α-MeDA and *N*-Me-α-MeDA) generates the corresponding *o*-quinone which can be trapped with nitrogen bionucleophiles such as *N*-acetyl-histidine and imidazole in a regioselective reaction that takes place predominantly at the 6-position of the catecholamine.



## 1. Introduction

The catecholamine dopamine (DA), **1** is an important neurotransmitter involved in the pathogenesis of Parkinson's disease (PD) [1,2], a degenerative neurological disorder characterized by rigidity, hypokinesia and tremor. A combination of factors have been proposed to explain this condition [3–7], in particular the formation of neurotoxic cysteinyl–catecholamine conjugates [8]. The primary metabolic pathway of catecholamines is oxidation to their catechol–quinones and semiquinones. These species are toxic intermediates that can cause cellular lesions at neutral pH via intermolecular and/or intramolecular Michael addition; they can also generate toxic reactive oxygen species (ROS) via redox cycling. Both mechanisms can lead to toxicity. Some authors reported the affinity of *N*-acetyldopamine (NADA, **2**) to nucleophilic groups in cuticular proteins of insects, with direct evidence of covalent binding obtained by solid-state NMR [9]. Moreover, DA and serotonin can produce covalent modification of cellular proteins, and possibly even cell degeneration, in the presence of high levels of metal ions [10]. Furthermore, both antitumor and toxic properties of several catecholamines have been attributed to the inhibition of critical enzymes (e.g., DNA polymerase) by quinone-mediated covalent binding [11]. Indeed, inactivation of enzymes/proteins by *o*-quinones is presumably due to nucleophilic addition reactions of sulfhydryl or amino groups present in these macromolecules to the quinones [12,13]. Some syntheses of sulfhydryl-dopamine conjugates, involving the enzymatic oxidation of DA, have been reported [14–17]. Other methods are more demanding, such as those involving the electrochemical generation of oxidized species [18–23]. An additional drawback of electrochemical methods is that the initially formed adducts are further oxidized to their corresponding quinones at the applied potential, leading to complex mixtures. Other strategies have relied on the ability of metal ions [e.g., Fe(II/III) or Mn(II)] to catalyze the oxidation of DA in the presence of L-cysteine [24]. 3,4-Methylenedioxymethamphetamine (MDMA or Ecstasy) is a widely abused, psychoactive recreational drug. There is growing evidence that the neurotoxic profile of MDMA is greatly dependent upon its hepatic metabolism, which produces highly reactive derivatives, such as catecholamines, catecholamine thioethers, and quinones. Our group has previously assessed the toxicity of MDMA and some of its catecholamines metabolites, namely α-MeDA (**3**), *N*-Me-α-MeDA (**4**) and their corresponding glutathione (GSH) and *N*-acetylcysteine (NAcCys) conjugates, in rat cortical neuronal cultures [25,26]. To better understand the role of specific catecholamines in biological processes, the synthesis and structural elucidation of catecholamines adducts of amino acids, nucleosides and small peptides is of utmost significance. However, the synthesis of catecholamine conjugates with nitrogen bionucleophiles is difficult, due to the less nucleophilic nature of these compounds when compared with GSH or NAcCys. Hawley and co-workers [27,28] reported the electrochemical oxidation of NADA- and *N*-α-alanyldopamine-generated quinones with two nitrogen-centered nucleophiles, imidazole (Imid) and *N*-acetyl-histidine (NAcHis), to model cuticular proteins containing histidinyl residues. They reported the predominant products from both nucleophiles to be C6 adducts

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of NADA and *N*-α-alanyldopamine. We describe herein for the first time the chemical and enzymatic synthesis of nitrogen adducts of relevant catecholamines (**1**–**4**).

## 2. Results

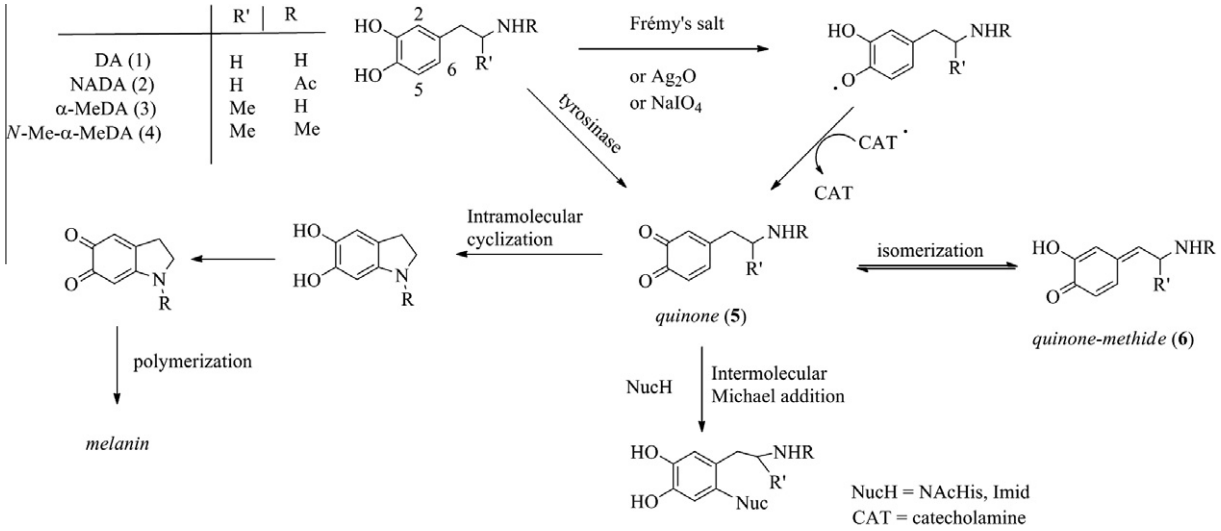
When subjecting **1** to enzymatic oxidation with tyrosinase using our previously reported method [25], followed by quenching of the respective *o*-quinone (**5**) with nitrogen nucleophiles, such as imidazole or the amino acids lysine (Lys), histidine (His), *N*-acetyllysine (NAcLys), NAcHis, and tryptophan (Trp) only intractable mixtures were obtained. Chemical oxidation with $NaIO_4$, $Ag_2O$ and Frémys's salt similarly produced a black residue, probably corresponding to melanin, a dark polymeric pigment stemming from intramolecular amine cyclization of the quinone and ensuing polymerization (Scheme 1). Tse et al. [29], reported that the intramolecular cyclization of **1** is faster than any intermolecular reaction, except when sulfur nucleophiles (e.g., Cys and GSH) are present. Indeed, the reaction of **1** with NAcCys was reported to be $10^6$-fold faster than with NAcHis [17]. In order to restrict this unwanted reaction we focused on **2**, where the presence of the *N*-acetyl protecting group was expected to prevent the pathway of intramolecular cyclization and subsequent polymerization. A further consideration was that the ionizable groups of Lys or His predominate in a zwitterionic form, involving protonated (and therefore non-nucleophilic) nitrogens, under our experimental conditions (pH 7.4). To ensure that the progress of the reactions would not be hampered by protonation of potentially nucleophilic sites, we used the *N*-protected amino acids NAcHis and NAcLys. The intermediate *o*-quinone **5** should in principle undergo a 1,4 or 1,6 intermolecular Michael addition in the presence of nucleophiles, although reaction on the external chain of the quinone-methide (**6**) could not be excluded (Scheme 1). The products from reaction with the nucleophiles NAcHis and Imid were found only in trace amounts. This may be attributed in part to the fact that the quinone intermediates are short-lived species. Indeed, we conducted a kinetic spectrophotometric study, which clearly indicated that isomerization of **5** ($\lambda$ 390 nm) to **6** ($\lambda$ 500 nm) occurs spontaneously (see ESI). These results agree with previous reports [30,31], although some authors observed a slower decomposition of NADA–quinone by NMR [31,32]. We had definite evidence that the rate of disappearance of **5** was concentration-dependent. Thus, the lifetime of **5** in 50 mM phosphate (pH 7.4) was 30 min at 0.47 μM but decreased to 3 min at 1.40 μM. Taking into consideration the short lifetime of the quinones, the nucleophiles were added to the reaction mixture 5 min after starting the generation of the *o*-quinone, regardless of the specific (enzymatic or chemical) oxidation procedure.

**Table 1**
Reaction conditions and yields for catecholamine conjugates with the nucleophiles NAcHis and Imid.

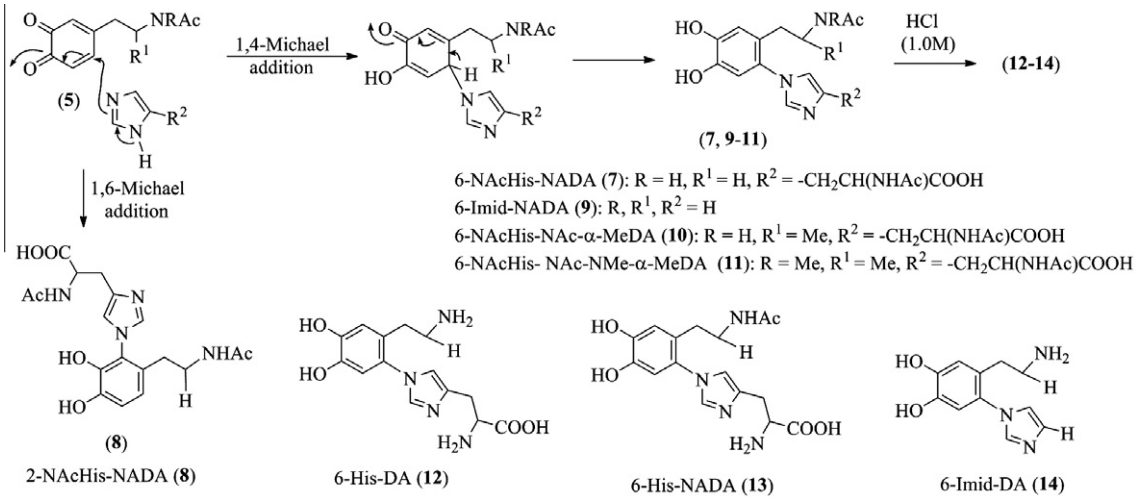
| Reaction conditions Oxidant/Nuc/catecholamine | Products | Isolated yield (%) |
|---|---|---|
| Ty/NAcHis (10 eq.)/**2**[a] | 2-NAcHis–NADA (**8**) | 2 |
| | 6-NAcHis–NADA (**7**) | 16 |
| $NaIO_4$/NAcHis (10 eq.)/**2** | 6-NAcHis–NADA (**7**) | 10 |
| Frémy's salt/NAcHis (5 eq.)/**2** | 6-NAcHis–NADA (**7**) | 4 |
| $Ag_2O$/NAcHis (5 eq.)/**2** | 6-NAcHis–NADA (**7**) | 2 |
| Ty/Imid (10 eq.)/**2** | 6-Imid–NADA (**9**) | 15 |
| $NaIO_4$/Imid (10 eq.)/**2** | 6-Imid–NADA (**9**) | 10 |
| $NaIO_4$/NAcHis (5 eq.)/**3** | 6-NAcHis–NAc-α-MeDA (**10**) | 5 |
| $NaIO_4$/NAcHis (10 eq.)/**4** | 6-NAcHis–NAc-NMe-α-MeDA (**11**) | 8 |

[a] Ty, tyrosinase.

An additional observation was that the yield of the catecholamine conjugate, 6-(*N*-acetyl)-histidine-*N*-acetyldopamine (6-NAcHis–NADA, **7**) could be improved (from trace amounts to 16%) if, prior to addition of the nucleophile, the enzyme was removed by gel chromatography filtration. Furthermore, these conditions allowed the isolation of a small amount of the isomer at the C2 position, 2-(*N*-acetyl)histidine-*N*-acetyldopamine (2-NAcHis–NADA, **8**) (Table 1). This procedure involving enzyme separation avoids further oxidation of the initially formed adducts, which are typically more prone to oxidation than the parent catechols [33]. As a further advantage, we measured the activity of the recovered enzyme and concluded that it remained active and thus reusable. The full structural characterization of the isolated reaction products showed that the nitrogen adducts resulted from a 1,4-Michael addition reaction to C6, the most electropositive site on the *o*-quinone ring (Scheme 2). This contrasts with the regioselectivity observed in reactions with sulfur nucleophiles, where the attack occurs predominantly at the C5 of the *o*-quinone although minor products resulting from attack at the C2 position were observed by some




**Scheme 1.** Proposed pathway for catecholamine metabolism, yielding reactive intermediates capable of alkylating cellular components or generating reactive oxygen species, ultimately leading to toxicity.




**Scheme 2.** Proposed mechanism for the conjugated Michael-addition of the nitrogen nucleophiles NAcHis and Imid, to biologically relevant catecholamines.

authors [15,17,23,34,35]. It has been proposed that base catalysis by the neighboring oxygen on C4 enhances the nucleophilicity of the sulfur compound for attack on C5 [36]. However, a similar argument (proximity of the oxygen on C3) might be used regarding attack on C2, which is minor with sulfur nucleophiles. This suggests that other factors (e.g., relative softness/hardness of the electrophilic sites on the quinones, stereochemical hindrance) may be at play. Interestingly, in contrast with 6-NAcHis–NADA (**7**), the NMR spectra of 2-NAcHis–NADA (**8**) indicated the presence of two conformers. We observed a partial, temperature-dependent and reversible, merging of the duplicated signals, consistent with restricted rotation, presumably through the histidine–NADA bond. This restriction may have been induced by an intramolecular hydrogen bond (e.g., involving the hydroxyl on C3 and the histidine nitrogen bound to NADA), which is not possible in adduct **7**. Compound **7** and 6-*N*-imidazole–*N*-acetyldopamine (6-Imid–NADA, **9**) have previously been reported as products from the electrochemical oxidation of **2** [27,28]. However, we question the structural characterization of **9** reported therein. Indeed, we performed a full characterization of **9** by two-dimensional NMR spectroscopy and found discrepancies regarding the reported assignments, namely a >1 ppm difference for the imidazole H2′ (cf. Electronic Supporting Information (ESI) for the spectroscopic data). From analysis of Table 1 it can be concluded that the best result was obtained with tyrosinase and the most discouraging one with $Ag_2O$. With $NaIO_4$ the yields were only slightly lower than with tyrosinase, suggesting that the mechanism for quinone formation, either via simultaneous transfer of two-electrons (tyrosinase) or consecutive transfer of one-electron with formation of a semiquinone intermediate does not affect the progress of the reaction (Scheme 1). Upon reaction of NADA-quinone with a series of other amino acids only adducts derived from reaction with valine and tryptophan could be detectable by electrospray ionization mass spectrometry (ESI-MS). Given the results obtained with NADA–quinone, and with the purpose of obtaining nitrogen adducts from the Ecstasy metabolites **3** and **4**, we generated the quinones from 3 and 4 with $NaIO_4$ and selected NAcHis as a representative, and biologically relevant, nitrogen nucleophile. In view of the instability of these compounds, which are prone to intramolecular amine cyclization of the quinone and subsequent polymerization when bearing a free amino group, the nitrogen was protected by acetylation prior to reaction with NAcHis. The adducts 6-(*N*-acetyl)-histidine–*N*-acetyl-α-methyldopamine (**10**) and 6-(*N*-acetyl)-histidine–*N*-acetyl-*N*-methyl-α-methyldopamine (**11**), formed by chemical oxidation with $NaIO_4$ of catecholamines **3** and **4** respectively, were obtained in modest yields (Table 1). Despite the difficulty found in the synthesis of these compounds, which arose from the instability of the *o*-quinone and lower reactivity of the nucleophiles, the method presented herein uses a less demanding protocol that those reported in the literature for other catecholamines [27,36–38] and allows the isolation of adducts in acceptable yields, amenable to complete structural analysis and future use as biomarkers. It should be noted that while some of the $^1$H NMR signals for adducts **10** and **11** were duplicated (cf. Section 4 and Supplementary material), this does not compromise their characterization. Indeed, since catechols **3** and **4** were used as racemates, both **10** and **11** were produced as mixtures of two diastereomers [6-(*N*-acetyl)-*S*-histidine-(*R*,*S*)-*N*-acetyl-α-methyldopamine and 6-(*N*-acetyl)-*S*-histidine-(*R*,*S*)-*N*-acetyl-*N*-methyl-α-methyldopamine, respectively], which were indistinguishable (and therefore unseparable) in our HPLC conditions. The *N*-acetyl protecting groups could be easily removed by hydrolysis, thus allowing the synthesis of the dopamine conjugates, otherwise impossible to achieve. Indeed, on hydrolysis under acidic conditions compounds **7**, **8**, and **9** gave the DA-nitrogen adducts 6-*N*-histidine-dopamine (6-His-DA, **12**), 6-*N*-histidine-*N*-acetyldopamine (6-His-NADA, **13**), and 6-*N*-imidazole-dopamine (6-Imid-DA, **14**), in 27%, 21%, and 40% yield, respectively (Scheme 2). Only 6-His-NADA (**13**) was identified as a partially deacetylated compound. We have not attempted any deacetylation of **8** due to the low yield of the isolated compound. Although the *S*-enantiomer of MDMA is more potent than the *R*-enantiomer in producing the typical effects that are characteristic of Ecstasy, MDMA and related compounds are consumed as racemates. Despite the limited information, the rates of elimination of both enantiomers are expected to be different [39]. Likewise, little is known about the relative susceptibilities of the enantiomers of **3** and **4** to enzyme-mediated oxidation to the corresponding quinones. A kinetic *in vitro* study with mushroom tyrosinase, using enantiomorphs of several diphenols (e.g., L-, D, and D,L-dopa; L-, D, and D,L-α-methyldopa), demonstrated that the enzyme has stereospecificity in its affinity towards the substrates (as measured by $K_m$) but not in the reaction rate ($V_{max}$) of those substrates [40]. It remains to be established whether stereospecific oxidation and adduct formation rates will occur *in vivo*.

## 3. Conclusion

We report herein for the first time the enzymatic and chemical synthesis of important catecholamine adducts with nitrogen nucleophiles using a less demanding protocol than those described

in the literature. Although the adducts were obtained in modest yields, full spectroscopic analysis was possible and proved that the reaction is regioselective, with the modification taking place predominantly at the 6-position of the catecholamines. Modification of peptides and proteins occurs in all eukaryotic systems and the complete elucidation of macromolecules modified by catecholamines is crucial to understand the role of catecholamines in several biological processes. Although mass spectrometric techniques are becoming routine in analyzing these modifications, detailed structural characterization remains difficult when dealing with unknown complex samples; toward that end, the availability of synthetic standards, such as those described in the present manuscript, is crucial. Should these adducts prove to be biologically relevant, their potential clinical use as (pre)disease biomarkers could represent an important breakthrough in the treatment and prevention of neurodegenerative diseases.

## 4. Experimental

### 4.1. Materials

NAcLys, NAcHis, Imid, DA (hydrochloride salt), and mushroom tyrosinase (5370 U/mg of solid; EC 1.14.18.1), were obtained from Sigma–Aldrich® (Steinheim, Germany) and used as received, unless specified. α-MeDA (**3**), and *N*-Me-α-MeDA (**4**) were synthesized and purified as described previously [25].

### 4.2. General procedure for the enzymatic oxidation of catecholamines, followed by addition of the nitrogen nucleophile

Mushroom tyrosinase (0.2 mg, 1074 units) was added to a solution of catecholamine (20 mg) in 1 mL phosphate buffer (pH 7.4, 50 mM) at r.t. The color changed to yellow, indicating the formation of the corresponding *o*-quinone. The enzyme was removed from the reaction mixture using a 5 mL HitrapTM desalting column (GE Heathcare®), eluted with water (20 mL). The yellow solution of catecholamine–quinone was then added dropwise to a solution of NAcHis (10 eq.) or Imid (10 eq.) in phosphate buffer (15 mL, pH 7.4, 50 mM), and the reaction mixture was stirred for 1 h. At the terminus of the reaction 1 mL of 88% formic acid was added, and the reaction mixture was carefully evaporated under reduced pressure. The resulting orange oil was dissolved in water and purified by reverse-phase (RP-18) modified silica gel column chromatography, using water (100 mL) and 50% MeOH (100 mL) as eluent. The fraction containing the product eluted with 50% MeOH and was purified by HPLC (see ESI).

#### 4.2.1. 6-NAcHis–NADA (*7*)

Yellow oil (6.4 mg, 16%). UV/vis ($\lambda_{max}$, nm): 286. $^1$H NMR ($D_2O$, δ): 1.78 (3H, s, $COCH_3$), 1.91 (3H, s, NAcHis-$COCH_3$), 2.36–2.44 (2H, m, H7), 3.05–3.15 (3H, m, H8 + NAcHis-Hβa), 3.30 (1H, dd, *J* = 15.4 and 5.0 Hz, NAcHis-Hβb), *ca.* 4.70 (NAcHis-Hα), 6.80 (2H, s, H2 + H5), 7.38 (1H, s, NAcHis-H5), 8.74 (1H, s, NAcHis-H2) ppm. $^{13}$C NMR ($D_2O$) δ: 22.1 (NADA-$COCH_3$ + NAcHis-$COCH_3$), 26.8 (NAcHis-Cβ), 29.7 (C7), 39.9 (C8), 52.2 (NAcHis-Cα), 114.7 (C5), 117.9 (C2), 123.5 (NAcHis-C5), 126.4 (C6), 127.5 (C1), 130.1 (NAcHis-C4), 135.9 (NAcHis-C2), 143.6 (C3/4), 146.6 (C3/4), 163.6 (NAcHis-CO), 173.7 (NAcHis-$COCH_3$), 174.3 (NADA-$COCH_3$) ppm. MS(ESI) m/z: 391 $[MH]^+$.

#### 4.2.2. 2-NAcHis–NADA (*8*)

Yellow oil (0.5 mg, 1.3%). Two conformers were detected by $^1$H NMR at room temperature, possibly due to restricted rotation induced by an intramolecular hydrogen bond). UV/vis ($\lambda_{max}$, nm): 283.5. $^1$H NMR ($D_2O$) δ: 1.77 and 1.78 (3H, s, $COCH_3$), 1.83 and 1.84 (3H, s, NAcHis-$COCH_3$), 2.12–2.23 and 2.39–2.42 (2H, m, H7), 2.74–2.99 (2H, m, NAcHis-Hβ), 3.01–3.14 (2H, m, H8), 4.28 (1H, dd, *J* = 8.6 and 5.2 Hz, NAcHis-Hα), 4.38 (1H, dd, *J* = 8.2 and 4.7 Hz, NAcHis-Hα′), 6.81 and 6.87 (1H, s, H6), 6.88 (1H, s, H5), 7.38 and 7.41 (1H, s, NAcHis-H5), 8.76 (1H, s, NAcHis-H2) ppm. $^{13}$C NMR ($D_2O$) δ: 22.6 ($COCH_3$ + NAcHis-$COCH_3$), 26.1 (NAcHis-Cβ), 30.3 (C7), 40.7 (C8), 52.8 (NAcHis-Cα), 116.5 (C6), 118.5 (C5), 119.2 (NAcHis-C5), 123.1 (C1), 127.9 (C2), 131.5 (NAcHis-C4), 135.8 (NAcHis-C2), 143.6 (C3/4), 146.7 (C3/4), 163.6 (NAcHis-CO), 173.8 (NAcHis-$COCH_3$ + $COCH_3$) ppm. MS(ESI) m/z: 391 $[MH]^+$.

#### 4.2.3. 6-Imid–NADA (*9*)

Yellow oil (4.0 mg, 15%). UV/vis ($\lambda_{max}$, nm): 229 and 283.5. $^1$H NMR ($D_2O$) δ: 1.78 (3H, s, $COCH_3$), 2.45 (2H, t, *J* = 6.6 Hz, H7), 3.11 (2H, t, *J* = 6.5 Hz, H8), 6.84 (1H, s, H5), 6.85 (1H, s, H2), 7.54 (2H, s, Imid-H5 + H4), 8.82 (1H, s, Imid-H2) ppm. $^{13}$C NMR ($D_2O$) δ: 22.1 ($COCH_3$), 29.6 (C7), 40.1 (C8), 114.7 (C5), 117.8 (C2), 120.1 (Imid-C4/5), 124.4 (Imid-C4/5), 126.4 (C6), 127.6 (C1), 135.9 (Imid-C2), 143.5 (C3/4), 146.6 (C3/4), 174.3 ($COCH_3$) ppm. MS(ESI) m/z: 284 $[M+Na]^+$, 262 $[MH]^+$.

### 4.3. General procedure for the chemical oxidation of catecholamines, followed by addition of the nitrogen nucleophile

#### 4.3.1. Using $NaIO_4$ or Frémy's salt as oxidant

To a solution of catecholamine (5.5 mg, 1 eq.) in 10 mL of phosphate buffer (pH 7.4, 50 mM) at r.t. was added 1 eq. of $NaIO_4$ or Frémy's salt (weighed under inert atmosphere) and then a solution of 5 eq. of the nucleophile in 2 mL phosphate buffer (pH 7.4, 50 mM). After stirring for 3 h the reaction was stopped by addition of 1 mL of 88% formic acid. The reaction mixture was carefully evaporated under reduced pressure and the compound purified by HPLC (see ESI).

#### 4.3.2. Using $Ag_2O$ as oxidant

To a solution of **2** (102 μmol) in 6 mL of DMF were added 10 eq. of $Ag_2O$. The color changed to yellow, indicating the formation of the corresponding *o*-quinone. After vortexing (5 min) to remove the excess of oxidant, the nucleophile, NAcHis (5 eq.) in 30 mL of 50 mM phosphate buffer (pH 7.4) was slowly added to the *o*-quinone solution. After stirring for 3 h the reaction was stopped by addition of 1 mL of 88% formic acid. The reaction mixture was carefully evaporated under reduced pressure, using toluene to help removing the DMF, and the compound was purified by HPLC (see ESI).

#### 4.3.3. 6-(*N*-acetyl)-histidine-(*R,S*)-*N*-acetyl-α-methyldopamine (*10*)

Yellow oil (0.5 mg, 5%, with $NaIO_4$). UV/vis ($\lambda_{max}$, nm): 284. $^1$H NMR ($D_2O$) δ: 0.99 (3H, d, *J* = 7.1 Hz, H9), 1.81 (3H, s, $COCH_3$), 1.99 (3H, s, NAcHis-$COCH_3$), 2.84 (2H, s, H7), 2.99 (2H, s, NAcHis-Hβ), 3.33 (1H, m, H8), 6.87 (2H, s, H2 + H5), 7.43 (1H, s, NAcHis-H5), 7.91 (1H, s, NAcHis-H5′), 8.80 (1H, s, NAcHis-H2), ppm. MS(ESI) m/z: 405 $[MH]^+$.

#### 4.3.4. 6-(*N*-acetyl)-histidine-(*R,S*)-*N*-acetyl-*N*-methyl-α-methyldopamine (*11*)

Yellow oil (0.8 mg, 8%, with $NaIO_4$). UV/vis ($\lambda_{max}$, nm): 223 and 284. $^1$H NMR ($D_2O$) δ: 0.99–1.02 (3H, m, Hz, H9), 1.15 (3H, m, H9′), 1.89 (3H, s, $COCH_3$), 1.97 (3H, s, NAcHis-$COCH_3$), 2.18 (2H, s, H7), 2.52 (2H, m, NAcHis-Hβ), 2.75 (3H, s, N-$CH_3$), 3.30 (1H, m, H8), 6.82 (2H, s, H2 + H5), 7.46 (1H, s, NAcHis-H5), 8.74 (1H, s, NAcHis-H2) ppm. MS(ESI) m/z: 419 $[MH]^+$.

### 4.4. General method for the hydrolysis of N-acetyldopamine conjugates

The catecholamine–nitrogen conjugate (28 μmol) was dissolved in 10 mL HCl (1.0 M), and stirred at 90 °C for 6 h. The solvent was then removed under vacuum and the products were isolated by HPLC (see ESI for more details).

#### 4.4.1. 6-His–DA (**12**)

Yellow oil (2.3 mg, 27%). UV/vis ($\lambda_{max}$, nm): 214 and 284. $^1$H NMR ($D_2O$) δ: 2.61 (2H, t, $J$ = 7.7 Hz, H7), 2.98 (2H, t, $J$ = 7.7 Hz, H8), 3.26 (1H, dd, $J$ = 15.4 and 7.4 Hz, His-Hβa), 3.35 (1H, dd, $J$ = 15.4 and 5.8 Hz, His-Hβb), 4.04 (1H, t, $J$ = 7.7 Hz, His-Hα), 6.89 (1H, s, H2/5), 6.90 (1H, s, H2/5), 7.48 (1H, s, His-H5), 8.82 (1H, s, His-H2) ppm. $^{13}$C NMR ($D_2O$) δ: 26.1 (His-Cβ), 27.6 (C7), 39.9 (C8), 53.7 (His-Cα), 115.1 (C5), 117.4 (C2), 122.9 (His-C5), 125.0 (C6), 126.3 (C1), 128.9 (His-C4), 136.4 (His-C2), 144.2 (C3/4), 147.1 (C3/4), 163.2 (His-CO) ppm. MS(ESI) m/z: 307 $[MH]^+$. HRMS-ESI m/z calcd. for $C_{14}H_{18}N_4O_4$ $[MH]^+$: 307.1401; obtained 307.1401.

#### 4.4.2. 6-His–NADA (**13**)

Yellow oil (2 mg, 21%). UV/vis ($\lambda_{max}$, nm): 211.4 and 284.1. $^1$H NMR ($D_2O$) δ: 1.78 (3H, s, $COCH_3$), 2.46 (2H, t, $J$ = 6.7 Hz, H7), 3.09 (2H, t, $J$ = 6.6 Hz, H8), 3.34 (2H, d, $J$ = 6.5 Hz, His-Hβ), 4.10 (1H, t, $J$ = 6.6 Hz, His-Hα), 6.84 (1H, s, H2/5), 6.85 (1H, s, H2/5), 7.48 (1H, s, His-H5), 8.81 (1H, s, His-H2) ppm. $^{13}$C NMR ($D_2O$) δ: 22.1 ($COCH_3$), 26.0 (His-Cβ), 29.7 (C7), 40.1 (C8), 55.4 (His-Cα), 114.8 (C5), 117.6 (C2), 123.1 (His-C5), 126.4 (C6), 127.5 (C1), 128.5 (His-C4), 136.5 (His-C2), 143.6 (C3/4), 146.7 (C3/4), 163.2 (His-CO), 174.3 ($COCH_3$) ppm. MS(ESI) m/z: 349 $[MH]^+$. HRMSESI m/z calcd. for $C_{16}H_{20}N_4O_5$ $[MH]^+$: 349.1512; obtained 349.1506.

#### 4.4.3. 6-Imid-DA (**14**)

Yellow oil (2.5 mg, 40%). UV/vis ($\lambda_{max}$, nm): 284. $^1$H NMR ($D_2O$) δ): 2.64 (2H, t, $J$ = 8.0 Hz, H7), 2.98 (2H, t, $J$ = 7.9 Hz, H8), 6.90 (1H, s, H2/5), 6.91 (1H, s, H2/5), 7.56 (2H, s, Imid-H5 + H4), 8.86 (1H, s, Imid-H2) ppm. MS(ESI) m/z: 220 $[MH]^+$. HRMSESI m/z calcd. for $C_{11}H_{13}N_3O_2$ $[MH]^+$: 220.1081; obtained 220.1081.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2012.05.002.

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