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Diamination by N-coupling using a commercial laccase

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ABSTRACT

Nuclear diamination of *p*-hydrobenzoquinones with aromatic and aliphatic primary amines was catalysed by an immobilised commercial laccase, Denilite® II Base, from Novozymes. The amine and the *p*-hydrobenzoquinone was reacted under mild conditions (at room temperature and at 35 °C) in a reaction vessel open to air in the presence of laccase and a co-solvent to afford, exclusively, the diaminated *p*-benzoquinone. These compounds may have potential antiallergic, antibiotic, anticancer, antifungal, antiviral and/or 5-lipoxygenase inhibiting activity.

1. Introduction

Laccases are a group of oxidative enzymes that are able to abstract hydrogen from phenolic hydroxyl groups by using molecular oxygen as an electron acceptor, resulting in phenoxy radicals that undergo a broad range of reactions.[1–3] Laccases catalyse the oxidation of organic compounds such as methoxyphenols, phenols, *o*- and *p*-diphenols, aminophenols, polyphenols, polyamines, and lignin-related molecules.[1–3] Laccase applications span from the textile to the pulp and paper industries, and from food applications to bioremediation processes.[2]

The high oxidative selectivity exhibited by laccases in aqueous solution has made them attractive for a variety of oxidations in green chemistry. As a result of these properties, their application as biocatalysts in organic chemistry has been investigated. Several examples have been reported in the literature such as the synthesis of actinocin[4] and cinnabarinic acid,[5] the synthesis of substituted triazolobenzothiadiazinones,[6] dimerization of various compounds such as estradiol,[7] penicillin X,[8] bisphenol A[9] salicyclic acids[10] synthesis of polymers,[11–13] oxidative coupling of hydroquinone and mithramicine[14] or (+)-catechin,[15] oxidation of substituted imidazoles.[16] derivatization of dihydrocaffeic acid,[17] L-tryptophan[18] and *para*-dihydroxylated compounds,[19] oxidative domino reactions of dibenzofuranones[20] and naphthoquinone synthesis.[21] Several antineoplastic drugs that are in use contain an aminoquinone moiety. The common antibiotic, Mitomycin, is an example of such a drug. Nakijiquinone-derivatives[22] and herbimycin-derivatives[23] are others that are under development. Reports of simple aminoquinones possessing activity against a number of cancer cell-lines,[24–26] antiallergic or 5-lipoxygenase inhibiting activity[27] has prompted research into new routes to the synthesis of aminoquinones.

In this paper we report on the synthesis of diaminobenzoquinones using the commercial laccase, Denilite II Base (on an inert support) from Novozymes. This laccase is from the *Aspergillus sp.* and is used for bleaching denim in the textile industry. The synthesis of diaminated benzoquinones, catalysed by a fungal laccase, has been reported previously, but on a smaller range of substrates.[28] This paper is, to the best of our knowledge, the first on the synthesis of diaminobenzoquinones using a commercial laccase on an inert support.

2. Results and discussion

The *p*-hydrobenzoquinones (**1a**–**d**) and primary amines (**2a**–**f**) used in this study are depicted in Figure 1.

It was anticipated that the reaction of a hydroquinone **1a**–**d** with a primary amine **2a**–**f** would afford a monoaminated benzoquinone **3** or a diaminated benzoquinone **4** or, both **3** and **4** (Scheme 1).

In our first approach, 1 equiv of primary amine was reacted with 1 equiv of *p*-hydroquinone in an attempt to obtain the monoaminated product **3**. The reactions were conducted at room temperature

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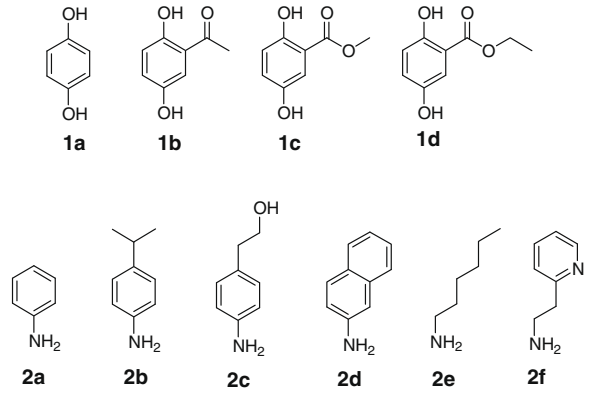


Figure 1. The *p*-hydrobenzoquinones and primary amines used in this study.

Table 1
Synthesised diaminated *p*-benzoquinones (yield in parentheses) at room temperature in 10% aqueous MeOH

Entry	Hydroquinone	Primary amine	Reaction time (h)	Method[a]	Diaminated product
1	**1a**	**2e**	116	A	**5** (2%)
2	**1a**	**2b**	95	A	**6** (32%)
3	**1b**	**2a**	171	A	**7** (11%)
4	**1b**	**2b**	95	A	**8** (14%)
5	**1b**	**2c**	90	A	**9** (4%)
6	**1c**	**2a**	172	A	**10** (26%)
7	**1c**	**2b**	144	A	**11** (20%)
8	**1c**	**2f**	142	A	**12** (16%)
9	**1c**	**2c**	149	A	**13** (11%)
10	**1d**	**2a**	172	A	**14** (33%)
11	**1d**	**2c**	148	A	**15** (19%)

[a] Method A—0.0750 g laccase, 1 equiv amine, MeOH (1.0 mL).

using a mixture of water, 10% methanol and succinate-lactate buffer (pH 4.5). No monoaminated products were obtained. The results for these reactions are summarised in Table 1.

From the analysis of the data for the isolated products of these reactions reported in Table 1 it was found that only the diaminated product was observed for each of these reactions despite the use of only 1 equiv of primary amine. The formation of the diaminated product accounts for the generally low yields (between 2% and 33%) observed for these reactions. A characteristic red or brown colour of the products was indicative of the formation of quinonamines and simplified product isolation. From the ^{1}H NMR spectrum of product **14** two aniline residues and one proton, H-6, for the quinone ring was observed (Fig. 2).

The amine proton is also observed, but no phenolic hydroxy groups. For the ^{13}C NMR spectrum, signals characteristic for quinones are observed in the 175–180 ppm range. The molecular ion peak of 361.1187 in the HRMS spectrum of **14** correlated with the calculated mass of 361.1188. This mass could be attributed to the amination of **1d** with two molecules of **2a** with the loss of six hydrogen atoms. The structures of the diaminated products from the reactions in Table 1 are shown in Figure 3.

From the results it was evident that at least 2 equiv of primary amine would be required in order to increase the yield of the diaminated product from these reactions. The conditions for these reactions also had to be optimised in order to speed up the formation of the product. For this purpose certain reactions were repeated and conducted at 35 °C using at least 2 equiv (Method B) and 3 equiv (Method C) and the total volume (5 mL) of these reactions was half of that used in Method A (10 mL). The solvent was also changed from 10% methanol to 20% dimethylformamide, a more polar solvent, to increase the solubility of the substrates.

Since the hydroquinone was observed by TLC for these reactions, it was concluded that the concentration of laccase was not sufficient to catalyse the conversion of all the hydroquinone to the quinone. In order to increase the yield, it was decided to conduct reactions with a threefold higher concentration of laccase (Methods D, E, F and G). It was also decided to increase the equivalents of the amine (Method F, 4 equiv) since this would promote the formation of the diaminated

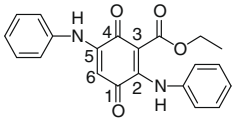
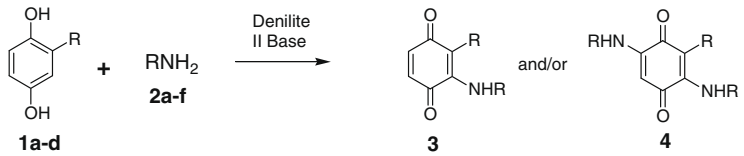


Figure 2. The structure of compound **14** with atom numbering.

products as discussed by Chakraborty et al.[29] The results for these reactions are summarised in Table 2.

From the data in Table 2 it can be seen that the yields range from moderate (entries 4 and 15) to poor (entries 1, 3 and 8) when the reactions are done at 35 °C. For **5** (entry 1) only a 4% increase in yield was observed upon heating using Method C for which previously only a 2% yield was observed at room temperature using Method A. The use of Method E, however, had increased the yield by almost fourfold (22% from 6%) and 11-fold (22% from 2%, Method A, Table 1) for **5**. For **17** a yield of only 5% was obtained (entry 3) upon heating. For **11** the yield only increased by 5% using Method G (entry 7). In the case of **15** the yield was increased by almost twofold using Method G (entry 10). For **13** the yield increased by almost fivefold (52% from 11%) using Method D (entry 15). Both methods B and D proved successful for synthesising **7**, but only afforded the product in 16% and 25% yield (entry 14), respectively. From the results in Table 2 it was concluded that the reactions did not go to completion and this may be attributed to the hydroquinone not being completely converted to the quinone. The structures of the diaminated products synthesised at 35 °C are shown in Figure 4.

As a consequence it was decided to investigate a set of reactions in which the quantity of the Denilite II Base was at least doubled (from 0.23 g to 0.48 g, Methods H and I). It was also decided to add the enzyme at different time intervals to ensure fresh enzyme and to circumvent the possibility of denaturing all at once. The results of this investigation are reported in Table 3 from which it is apparent that doubling the quantity of enzyme does increase the



R = H, $COCH_3$, CO_2CH_3, $CO_2CH_2CH_3$,

Scheme 1.

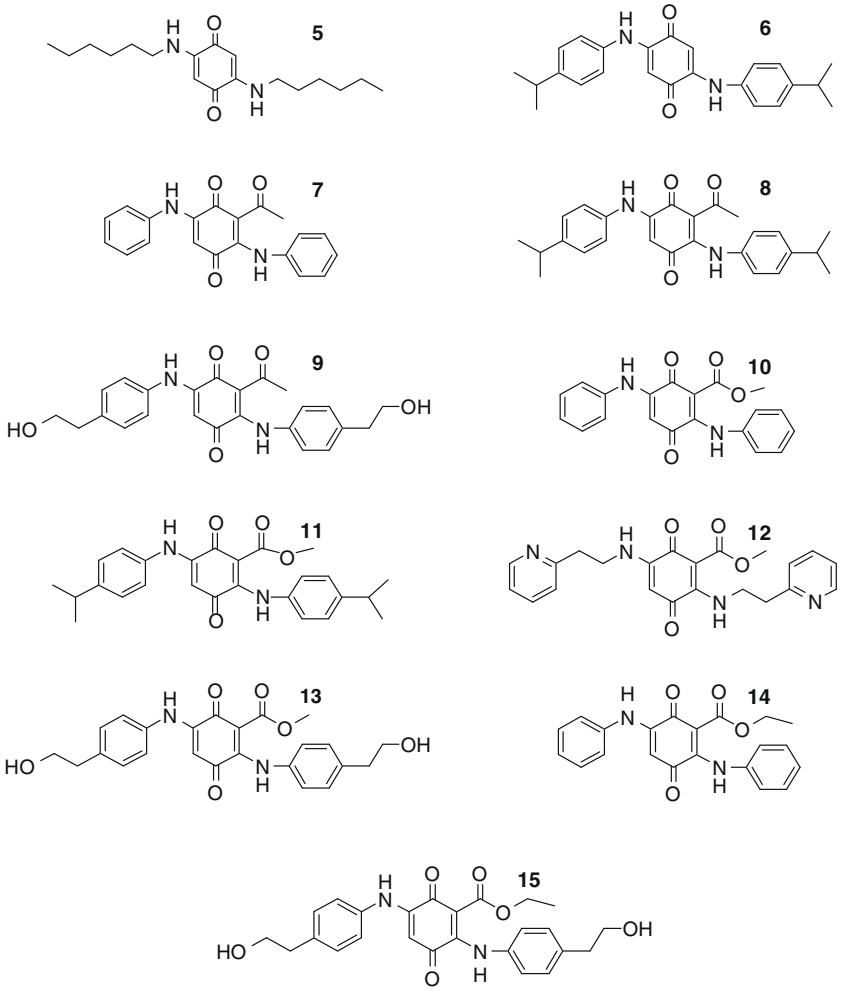


Figure 3. Structures of diaminated products **5**–**15** synthesised at room temperature.

Table 2
Synthesised diaminated *p*-benzoquinones (yield in parentheses) at 35 °C in 20% aqueous DMF

Entry	Hydroquinone	Primary amine	Reaction time (h)	Method[a]	Diaminated product
1	**1a**	**2e**	72	C	**5** (6%)
			72	E	**5** (22%)
2	**1b**	**2a**	96	B	**16** (16%)
3	**1b**	**2e**	68	C	**17** (5%)
4	**1b**	**2b**	96	B	**8** (52%)
5	**1c**	**2d**	96	F	**18** (20%)
6	**1c**	**2e**	72	E	**19** (19%)
7	**1c**	**2b**	72	G	**11** (25%)
8	**1d**	**2e**	96	E	**20** (12%)
9	**1d**	**2b**	72	G	**21** (21%)
10	**1d**	**2c**	72	G	**15** (36%)
11	**1b**	**2f**	92	C	**22** (20%)
12	**1c**	**2f**	96	C	**12** (21%)
13	**1d**	**2f**	96	C	**23** (32%)
14	**1b**	**2a**	96	B	**7** (16%)
			72	D	**7** (25%)
15	**1c**	**2c**	72	D	**13** (52%)

[a] Method B—0.075 g laccase, 2 equiv amine, DMF (1.0 mL). Method C—0.075 g laccase, 3 equiv amine, DMF (1.0 mL). Method D—0.23 g laccase, 2 equiv amine, DMF (1.0 mL). Method E—0.23 g laccase, 3 equiv amine, DMF (1.0 mL). Method F—0.23 g laccase, 4 equiv amine, DMF (1.5 mL). Method G—0.23 g laccase, 2 equiv amine, DMF (1.0 mL).

yield of the products **5**, **9**, **12** and **22** (entries 2–5) except for **5** (entry 1) for which the yield has decreased by 7%.

For **7** there has only been a slight increase in yield, 7% (entry 2), when compared to Method D. A twofold increase (16%) for **7** (entry 2) is observed when compared to Method B. Similarly for **13** and **21**, a 6% and 9% yield increase was observed when compared to using Method D. A sevenfold increase in yield (28%, previously 4%) is observed for **9** when compared to using Method A.

In accordance with the literature,[30,31] the second substitution of monoaminated substances usually take place *para* to the first amination site. In these compounds, C2 is believed to be the first amination site and C5 the second. The diaminated products have a characteristic aromatic proton peak in the 5.7–6.6 ppm region for the H6/H3 proton in the ^{1}H NMR spectrum and a characteristic peak in the region 90–100 ppm in the ^{13}C spectrum. This downfield shift is attributed to the deshielding of the H5/H3 nucleus caused by the electron withdrawing nitrogen and oxygen substituents on the ring. A proposed mechanism for nuclear diamination is shown in Figure 5.

It is believed that the role of laccase is simply that of an oxidant, three laccase oxidations occur before the diaminoquinones are formed. It is assumed that the second amination is kinetically faster than the first due to ring activation by the first amination.

Niedermeyer et al. has reported on the synthesis of monoaminoquinones and diaminobenzoquinones using fungal laccases (EC 1.10.3.2) from *Trametes spec.* and *Myceliophthora thermophila*.[28] Only four diaminobenzoquinones were synthesized using a molar ratio of up to 5:1 of primary amine to hydroquinone in the absence of a co-

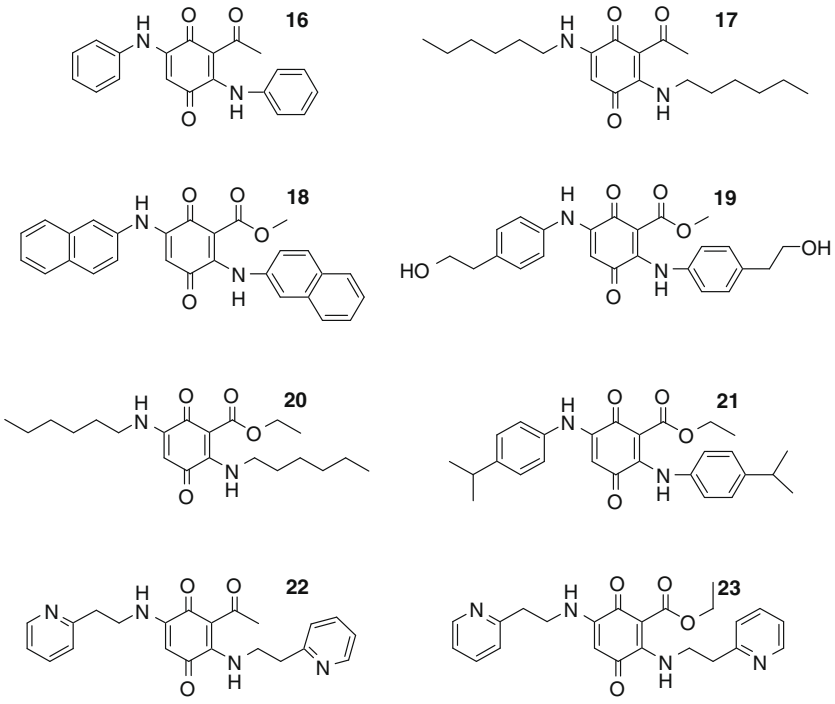


Figure 4. Structures of diaminated products **16**–**23** synthesised at 35 °C.

Table 3
Synthesised diaminated *p*-benzoquinones at 35 °C in 20% aqueous DMF

Entry	Hydroquinone	Primary amine	Reaction time (h)	Method[a]	Diaminated product
1	**1a**	**2e**	72	H	**5** (15%)
2	**1b**	**2a**	72	H	**7** (32%)
3	**1b**	**2c**	72	I	**9** (28%)
4	**1c**	**2c**	72	I	**13** (58%)
5	**1d**	**2b**	72	I	**21** (30%)

[a] Method H—0.48 g laccase, 5 equiv amine, DMF (1.0 mL). Method I—0.48 g laccase, 3 equiv amine, DMF (1.0 mL).

solvent in sodium acetate buffer, pH 5 (laccase from *T. spec.*, final activity 0.15 units mL^{-1}) or citrate-phosphate buffer, pH 7 (laccase from *M. thermophila*, final activity 1.0 units mL^{-1}). For this method the formation of mono-aminobenzoquinones was favoured as well as mixtures of the latter and the diaminobenzoquinone.

Chemical methods for nuclear amination have been reported in the literature, but these reports are few due to the susceptibility of the amino group to oxidation and hydrolysis. One method involves first halogenating (iodinating) the *p*-benzohydroquinone followed by coupling to the primary amine using a palladium catalyst and a triphenylphosphine ligand while refluxing under argon.[29] A drawback of this method is that it affords a mixture of monoaminated and diaminated benzoquinones. Another method involves the reaction of the iodinated *p*-benzohydroquinone with the primary amine at room temperature in a vessel open to air. A drawback of this method is that a mixture of iodinated monoaminated benzoquinone, monoaminated benzoquinone and diaminated benzoquinone is obtained.[29]

Well-known chemical oxidants that have been used to achieve nuclear amination of simple *p*-hydroquinones with simple primary aromatic amines are cupric acetate, silver (I) oxide and sodium iodate.[32] Amination can be accomplished more conveniently using sodium iodate.[32]

In summary, the one-pot, three-step biocatalytic method allows direct access to diaminobenzoquinones from the *p*-hydrobenzoquinones and affords only the diaminated product. This method has eliminated the use of an iodinated intermediate, a palladium catalyst and a phosphine ligand. While the laccase, Denilite II Base, may not be the ideal enzyme for these reactions, there is potential for the development of other laccase enzymes (preferably with high enzyme activity) to be used for the synthesis of diaminobenzoquinones.

3. Conclusions

The commercial laccase, Denilite II Base, can be used to access diaminobenzoquinones starting from *p*-dihydroxylated benzoic acid derivatives and aryl and alkyl primary amines. The yields of products from the reaction of primary aryl amines with *p*-dihydroxylated benzoic acid derivatives are generally higher than those from the reaction with alkyl primary amines. The formation of product is affected by factors such as the nucleophilicity of the amines, the electrophilicity of the hydroquinones, number of equivalents of amine, solubility and temperature.

4. Experimental section

4.1. General

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Mercury 200 MHz spectrometer. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded on the same instruments at 50 MHz. Chemical shifts are reported in ppm relative to the solvent peaks. High-resolution mass spectra were recorded on a Waters HPLC coupled to a Synapt HDMS mass spectrometer. Reactions were monitored by thin layer chromatography (TLC) on aluminium-backed Merck silica gel 60 F_{254} plates. Gravity column chromatography was done using Merck Silica Gel 60 (70–230 mesh). Melting points were determined using a Glassco melting point apparatus and are uncorrected.

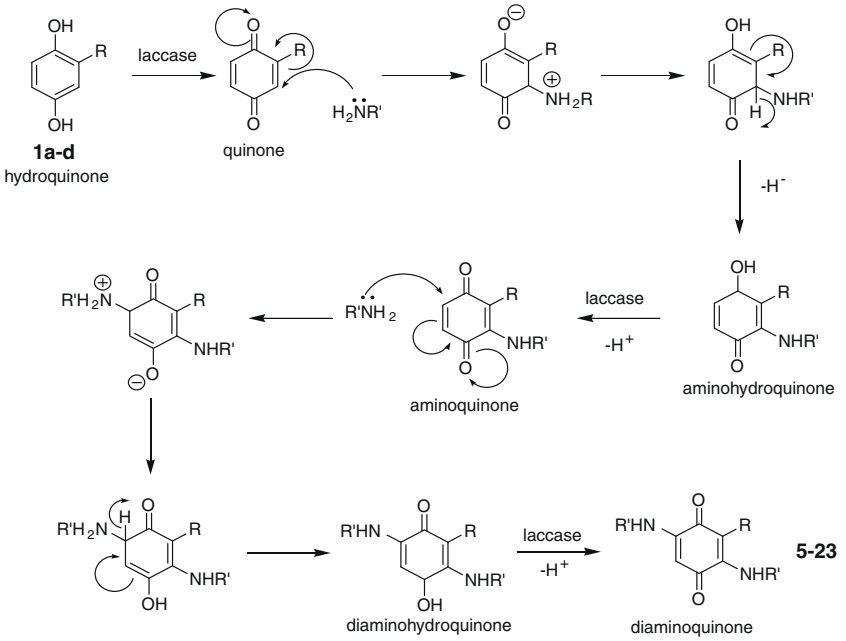


Figure 5. A proposed mechanism for nuclear diamination.

4.2. Materials

All chemicals were reagent grade materials.

4.2.1. Substrates

The *p*-hydroquinones and the primary amines were obtained from Sigma–Aldrich South Africa.

4.2.2. Enzymes

Myceliophthora thermophilia laccase was obtained from Novozymes as Denilite® II Base (800 U g^{-1}) on an inert carrier.

4.3. Diamination of 2,5-dihydroxybenzoic acid derivatives with aromatic and aliphatic primary amines

The following methods were used for the synthesis of the diaminated products.

4.3.1. Method A

The primary amine (0.3 mmol, 1 equiv) was added to a mixture containing 2,5-dihydroxybenzoic acid derivative (0.3 mmol), succinate-lactate buffer (2.0 mL, 1.0 M, pH 4.5), water (7.0 mL), MeOH (1.0 mL) and Denilite II Base (0.075 g). The reaction mixture was stirred at room temperature and monitored by TLC. After stirring, the solvent was removed on the rotary evaporator to afford a dark-brown residue that was purified by flash chromatography.

4.3.2. Method B

The primary amine (0.6 mmol, 2 equiv) was added to a mixture containing 2,5-dihydroxybenzoic acid derivative (0.3 mmol), succinate-lactate buffer (1.0 mL, 1.0 M, pH 4.5), water (3.0 mL), DMF (1.0 mL) and Denilite II Base (0.075 g). The reaction mixture was heated at 35 °C and monitored by TLC. After stirring, the solvent was removed on the rotary evaporator to afford a dark-brown residue that was purified by flash chromatography.

4.3.3. Method C

The same as Method B, except that 3 equiv (0.9 mmol) of primary amine was used.

4.3.4. Method D

The same as Method B, except that three times the amount of enzyme was used.

4.3.5. Method E

The same as Method B, except that 3 equiv (0.9 mmol) of primary amine and three times the amount of enzyme was used.

4.3.6. Method F

The same as Method E, except that DMF (1.5 mL) and 4 equiv (1.2 mmol) of primary amine was used.

4.3.7. Method G

The primary amine (0.6 mmol, 2 equiv) was added to a mixture containing the 2,5-dihydroxybenzoic acid derivative (0.3 mmol), succinate-lactate buffer (1.0 mL, 1.0 M, pH 4.5), water (3.0 mL), DMF (1.0 mL) and Denilite II Base (0.23 g). The reaction mixture was heated at 35 °C and monitored by TLC. After stirring, the solvent was removed on the rotary evaporator to afford a dark-brown residue that was purified by flash chromatography.

4.3.8. Method H

The primary amine (1.5 mmol, 5 equiv) was added to a mixture containing 2,5-dihydroxybenzoic acid derivative (0.3 mmol), succinate-lactate buffer (1.0 mL, 1.0 M, pH 4.5), water (3.0 mL), DMF (1.0 mL) and Denilite II Base (0.08 g). The reaction mixture was heated and stirred at 35 °C and after 1 h more Denilite II Base (0.08 g) was added and after an additional hour an additional Denilite II Base (0.08 g) was added. After 15 h Denilite II Base (0.24 g) was added at 1 h intervals in 0.08 g quantities. The total mass of Denilite II Base that was added is 0.48 g. After 72 h the solvent was removed on the rotary evaporator to afford a dark-brown residue that was purified by flash chromatography.

4.3.9. Method I

The same as Method H except that 3 equiv (0.9 mmol) of the primary amine was used.

4.4. 2,5-Bis-hexylamino-[1,4]benzoquinone 5

4.4.1. Method A

Stirring time = 116 h. Purification by flash chromatography (silica/$CHCl_3$–MeOH, 80:1) to afford a dark-brown solid (0.0017 g, 2.0%). R_f = 0.31 (MeOH/$CHCl_3$, 1:19).

4.4.2. Method B

Stirring time = 72 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:5) to afford an orange solid (0.0052 g, 6%). R_f = 0.29 (EtOAc/hexane, 1:4).

4.4.3. Method E

Stirring time = 94 h. Purification by flash chromatography (silica/MeOH–$CHCl_3$, 1:100) to afford a dark-brown solid (0.0134 g, 22%). (**M+H**$^+$ Found: 307.2390. $C_{18}H_{31}N_2O_2$ requires M+H, 307.2386); R_f = 0.15 ($CHCl_3$). Mp = 114–116 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.87 (6H, t J = 1.8, and 5.0 Hz, 2 × CH_3), 1.30 (10H, br s, 5 × CH_2), 1.50–1.80 (6H, m, 3 × CH_2), 3.13 (4H, q J = 6.0, 6.8 and 7.0 Hz, 2 × CH_2), 5.29 (1H, s, ArH) and 6.58 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.0, 22.5, 26.7, 28.2, 31.4, 42.6, 92.6, 151.3 and 178.1.

4.4.4. Method H

Stirring time = 72 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:8, 1:6, 1:4) to afford an orange-red crystalline solid (0.0147 g, 15%). R_f = 0.37 (EtOAc/hexane, 1:4).

4.5. 2,5-Bis-(4-isopropyl-phenylamino)-[1,4]benzoquinone 6

4.5.1. Method A

Stirring time = 95 h. Purification by flash chromatography (silica/$CHCl_3$) to afford a dark-brown solid (0.0360 g, 32%). (**M+H**$^+$ Found: 375.2088. $C_{24}H_{24}N_2O_2$ requires M+H 375.2073. R_f = 0.34 ($CHCl_3$). Mp = 241–242 °C [lit.[33] 189.5–190 °C]. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.28 (12H, m, 4 × CH_3), 2.95 (2H, 2 × CH), 6.05 (1H, s, 2 × ArH), 7.15–7.40 (8H, m, ArH) and 8.11 (2H, s, 2 × NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 24.2, 34.0, 95.7, 123.0, 127.8, 135.0, 147.3 and 180.3.

4.6. 3-Acetyl-2,5-bis-phenylamino-[1,4]benzoquinone 7

4.6.1. Method A

Stirring time = 171 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:4) to afford an orange-brown solid (0.0110 g, 11%). R_f = 0.32 ($CHCl_3$).

4.6.2. Method B

Stirring time = 96 h. Purification by flash chromatography (silica/$CHCl_3$; MeOH/$CHCl_3$, 1:19, 3:7, 2:3, 1:1) to afford an orange–brown solid (0.0144 g, 16%). (**M+H**$^+$ Found: 333.1228. $C_{20}H_{16}N_2O_3$ requires M+H 333.1229); R_f = 0.15 (MeOH/$CHCl_3$, 1:9). Mp = 169–171 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 2.68 (3H, s, CH_3), 6.07 (1H, s, ArH) 7.00–7.60 (10H, m, ArH) and 8.19 (2H, s, 2 × NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 32.9, 99.3, 123.0, 124.5, 126.4, 127.3, 129.3, 130.0, 137.3, 139.4, 146.6, 178.1 and 178.8.

4.6.3. Method D

Stirring time = 72 h. Purification by flash chromatography (silica/$CHCl_3$; MeOH/$CHCl_3$, 1:100, 3:100; 5:100) to afford a dark-brown solid (0.0252 g, 25%). (**M+H**$^+$ Found: 333.1234. $C_{20}H_{16}N_2O_3$ requires M+H, 333.1239); R_f = 0.09 ($CHCl_3$).

4.6.4. Method H

Stirring time = 72 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:9, 1:8) to afford an orange-red crystalline solid (0.0329 g, 32%).

4.7. 3-Acetyl-2,5-bis-(4-isopropyl-phenylamino)-[1,4]benzoquinone 8

4.7.1. Method A

Stirring time = 95 h. Purification by flash chromatography (silica/$CHCl_3$–hexane, 3:1) to afford a red-brown solid (0.0180 g, 14%). R_f = 0.28 ($CHCl_3$).

4.7.2. Method B

Stirring time = 96 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:9) to afford a dark-brown solid (0.0650 g, 52%). (**M+H**$^+$ Found: 417.2161. $C_{26}H_{26}N_2O_3$ requires M+H, 417.2178); R_f = 0.35 (EtOAc/hexane, 1:9). Mp = 139–141 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.15–1.4 (12H, m, 4 × CH_3), 2.64 (3H, br s, CH_3), 2.80–3.10 (2H, m, 2 × CH), 6.01 (1H, s, ArH), 7.00–7.30 (8H, m, ArH) and 8.16 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 23.9, 32.6, 33,8, 122.7, 124.0, 127.0, 127.6, 134.6, 136.8, 146.6, 147.1, 147.8, 177.8, 178.4, 191.8 and 200.7.

4.8. 3-Acetyl-2,5-bis-(4-(-2-hydroxy-ethyl-phenylamino)-[1,4]benzoquinone 9

4.8.1. Method A

Stirring time = 90 h. Purification by flash chromatography (silica/$CHCl_3$–hexane, 3:1) to afford a black solid (0.0050 g, 4%). R_f = 0.43 ($CHCl_3$).

4.8.2. Method I

Stirring time = 72 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:1; EtOAc; EtOAc/MeOH, 9:1) to afford a black solid (0.0377 g, 28%). (**M+H**$^+$ Found: 419.1601. $C_{23}H_{19}N_2O_6$ requires M+H 419.1243); R_f = 0.29 (EtOAc). Mp = 160–162 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 2.65 (3H, s, CH_3), 2.88 (4H, t J = 6.0 and 12.2 Hz, 2 × CH_2), (4H, t J = 6.4 and 6.6 Hz, 2 × CH_2), 6.00 (1H, s, ArH), 7.00–7.40 (8H, m, ArH) and 8.18 (1H, s, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 32.7, 38.6, 38.8, 63.4, 63.5, 122.9, 124.4, 126.4, 129.7, 129.3, 130.3, 135.4, 136.9, 137.6, 139.4, 146.4, 177.8 and 178.4.

4.9. 3,6-Dioxo-2,5-bis-phenylamino-cyclohexa-1,4-dienecarboxylic acid methyl ester 10

4.9.1. Method A

Stirring time = 172 h. Purification by flash chromatography (silica/$CHCl_3$) to afford a dark-brown solid (0.0270 g, 26%). (Found: **M**−**H**$^+$ 347.1027. $C_{20}H_{16}N_2O_6$ requires M−H 347.1032); R_f = 0.13 ($CHCl_3$). Mp = 185–187 °C [lit.[33] 202–203 °C]. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 3.16 (3H, s CH_3), 6.11 (1H, s, ArH), 7.15–7.60 (10H, m, ArH), 8.19 (1H, br s, NH) and 8.72 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 51.4, 95.9, 123.0, 123.8, 126.5, 127.14, 129.34, 129.7, 136.7, 137.3, 146.7, 177.0 and 178.6.

4.10. 2,5-Bis-(4-isopropyl-phenylamino-3,6-dioxo-cyclohexa-1,4-dienecarboxylic acid methylester 11

4.10.1. Method A

Stirring time = 144 h. Purification by flash chromatography (silica/$CHCl_3$–hexane, 1:1; MeOH/$CHCl_3$, 1:20) to afford a brown solid (0.0180 g, 20%). R_f = 0.64 (MeOH/$CHCl_3$, 1:19).

4.10.2. Method G

Stirring time = 72 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:6; 1:3; 1:1) to afford a dark-brown powder (0.0325 g, 25%). (**M+H**$^+$ Found: 433.2112. $C_{26}H_{28}N_2O_4$ requires

M+H, 447.2284); R_f = 0.31 (Et_2O/hexane, 1:1). Mp = 189–191 °C. ^1H NMR (200 MHz, $CDCl_3$): δ = 1.15–1.35 (12H, m, 4 × CH_3), 2.85–3.05 (2H, m, 2 × CH), 3.12 (3H, s, CH_3), 6.06 (1H, s, ArH), 7.00–7.35 (8H, m, ArH), 8.17 (1H, br s, NH) and 8.67 (1H, s, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 23.9, 33.7, 33.8, 51.3, 95.6, 109.7, 123.1, 123.8, 127.3, 127.6, 134.4, 134.9, 147.0, 147.5, 148.2, 177.0 and 178.5.

4.11. 3,6-Dioxo-2,5-bis-(2-pyridin-2-yl-ethylamino)-cyclohexa-1,4-dienecarboxylic acid methyl ester 12

4.11.1. Method A

Stirring time = 142 h. Purification by flash chromatography (silica/MeOH–$CHCl_3$, 1:19, 3:7, 2:3, 1:1) to afford a dark-brown solid (0.0200 g, 16%). R_f = 0.48 (MeOH/$CHCl_3$, 1:9).

4.11.2. Method C

Stirring time = 142 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:2; EtOAc; MeOH/EtOAc, 1:19) to afford an orange–brown solid (0.0260 g, 21%). (**M**+**H**$^+$ Found: 407.1733. $C_{22}H_{22}N_4O_4$ requires M+H 407.1719); R_f = 0.19 (MeOH/EtOAc, 1:19). Mp = 93–95 °C. ^1H NMR (200 MHz, $CDCl_3$): δ = 3.10 (4H, t J = 6.2 and 6.4 Hz, CH_2), 3.62 (4H, q J = 6.0, 6.2 and 6.4 Hz, CH_2), 3.85 (3H, m, CH_3), 4.41 (1H, m, NH), 5.46 (1H, s, ArH), 7.00–7.35 (4H, m, ArH), 7.55–7.70 (2H, m, ArH), 8.08 (1H, br s, NH) and 8.56 (2H, s, ArH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 36.1, 36.6, 42.0, 43.8, 52.5, 93.2, 122.2, 123.4, 123.5, 136.9, 138.5, 148.2, 149.8, 149.9, 158.0, 176.2 and 176.6.

4.12. 2,5-Bis-(4-(2-hydroxyl-ethyl)-phenylamino]-3,6-dioxo-cyclohexa-1,4-diene carboxylic acid methylester 13

4.12.1. Method A

Stirring time = 149 h. Purification by flash chromatography (silica/$CHCl_3$, MeOH/$CHCl_3$, 1:40, 1:20) to afford a black solid (0.0146 g, 11%). R_f = 0.18 (MeOH/$CHCl_3$, 1:19).

4.12.2. Method D

Stirring time = 72 h. Purification by flash chromatography (silica/$CHCl_3$; MeOH/$CHCl_3$, 1:100, 3:100; 5:100) to afford a black solid (0.0610 g, 52%). (**M**−**H**$^+$ Found: 435.1552. $C_{24}H_{24}N_2O_6$ requires M−H, 435.1556); R_f = 0.28 ($CHCl_3$). Mp = 183–185 °C. ^1H NMR (200 MHz, $CDCl_3$): δ = 2.91 (4H, m, 2 × CH_2), 3.21 (3H, s, CH_3), 3.90 (4H, m, 2 × CH_2), 6.10 (1H, s, ArH), 7.05–7.4 (8H, m, ArH) and 8.18 (1H, s, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 38.6, 38.7, 51.4, 63.3, 95.8, 123.2, 124.0, 128.8, 129.9, 130.3, 130.9, 135.1, 135.6, 137.3, 138.1, 146.8, 177.0 and 178.6.

4.12.3. Method I

Stirring time = 72 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:1; EtOAc; EtOAc/MeOH, 9:1) to afford a black solid (0.0767 g, 58%). R_f = 0.23 (EtOAc).

4.13. 3,6-Dioxo-2,5-bis-phenylamino-cyclohexa-1,4-dienecarboxylic acid ethyl ester 14

4.13.1. Method A

Stirring time = 90 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:4) to afford a dark-brown solid (0.0360 g, 33%). (**M**−**H**$^+$ Found: 361.1177. $C_{21}H_{18}N_2O_4$ requires M−H 361.1188); R_f = 0.64 (MeOH/$CHCl_3$; 1:49). Mp = 162–163 °C [lit.[34] 178–179 °C]. ^1H NMR (200 MHz, $CDCl_3$): δ = 1.02 (3H, t, J = 7.0 and 7.2 Hz, CH_3), 3.57 (2H, q J = 7.0 and 7.4 Hz, CH_2), 6.13 (1H, s, ArH), 7.20–7.55 (10H, m, ArH), 8.21 (1H, s, NH) and 8.72 (1H, s, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.1, 61.2, 96.1, 123.3, 124.0, 126.7, 127.4, 129.5, 130.0, 137.0, 137.6, 138.5, 146.9, 164.5, 177.4 and 179.0.

4.14. 2,5-Bis-[4-(2-hydroxy-ethyl)-phenylamino]-3,6-dioxo-cyclohexa-1,4-dienecarboxylic acid ethyl ester 15

4.14.1. Method A

Stirring time = 148 h. Purification by flash chromatography (silica/$CHCl_3$; MeOH/$CHCl_3$, 1:40, 1:20) to afford a dark-brown solid (0.0256 g, 19%). R_f = 0.52 (MeOH/$CHCl_3$, 1:9).

4.14.2. Method G

Stirring time = 72 h. Purification by flash chromatography (silica/MeOH–$CHCl_3$, 1:100) to afford a dark-brown solid (0.0489 g, 36%). (**M**+**H**$^+$ Found: 451.1857. $C_{25}H_{26}N_2O_6$ requires M+H, 451.1869); R_f = 0.29 (MeOH/$CHCl_3$, 1:19). Mp = 154–156 °C. ^1H NMR (200 MHz, $CDCl_3$): δ = 1.01 (2H, t, J = 7.0 and 8.0 Hz, CH_2), 2.87 (4H, m, 2 × CH_2), 3.57 (3H, q, J = 7.0 and 7.2 Hz, CH_2), 3.87 (4H, m, 2 × CH_2), (6.07 (1H, s, ArH), 7.00–7.20 (8H, m, ArH), 8.18 (1H, s, NH) and 8.70 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 13.8, 38.6, 38.7, 61.0, 63.4, 95.7, 123.1, 123.8, 129.8, 130.3, 135.0, 135.6, 137.2, 138.0, 146.7, 177.0 and 178.6.

4.15. 3-Acetyl-2,5-bis-phenylamino-[1,4]benzoquinone 16

4.15.1. Method A

Stirring time = 171 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:4) to afford an orange-brown solid (0.0110 g, 11%). R_f = 0.32 ($CHCl_3$).

4.15.2. Method B

Stirring time = 96 h. Purification by flash chromatography (silica/$CHCl_3$; MeOH/$CHCl_3$, 1:19, 3:7, 2:3, 1:1) to afford an orange–brown solid (0.0144 g, 16%). (**M**+**H**$^+$ Found: 333.1228. $C_{20}H_{16}N_2O_3$ requires M+H 333.1229); R_f = 0.15 (MeOH/$CHCl_3$, 1:9). Mp = 169–171 °C. ^1H NMR (200 MHz, $CDCl_3$): δ = 2.68 (3H, s, CH_3), 6.07 (1H, s, ArH) 7.00–7.60 (10H, m, ArH) and 8.19 (2H, s, 2 × NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 32.9, 99.3, 123.0, 124.5, 126.4, 127.3, 129.3, 130.0, 137.3, 139.4, 146.6, 178.1 and 178.8.

4.15.3. Method D

Stirring time = 72 h. Purification by flash chromatography (silica/$CHCl_3$; MeOH/$CHCl_3$, 1:100, 3:100; 5:100) to afford a dark-brown solid (0.0252 g, 25%). (**M**+**H**$^+$ Found: 333.1234. $C_{20}H_{16}N_2O_3$ requires M+H, 333.1239); R_f = 0.09 ($CHCl_3$).

4.15.4. Method H

Stirring time = 72 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:9, 1:8) to afford an orange-red crystalline solid (0.0329 g, 32%).

4.16. 3-Acetyl-2,5-bis-hexylamino-[1,4]benzoquinone 17

4.16.1. Method B

Stirring time = 68 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:4) to afford a brown solid (0.0049 g, 5%). R_f = 0.17 (EtOAc/hexane, 1:4). Mp = 33–35 °C. (Found: **MH**$^+$ 349.2485. $C_{20}H_{32}N_2O_3$ requires *MH* 349.2491). δ = ^1H NMR (200 MHz, $CDCl_3$): δ = 0.89 (6H, m, 2 × CH_3), 1.32 (10H, m, 5 × CH_2), 1.67 (6H, m, 3 × CH_2), 2.60 (3H, s, CH_3, 3.13 (2H, q J = 6.0 and 6.8 Hz, 2 × CH_2), 3.99 (2H, q J = 5.4, 6.8 and 7.2 Hz, 2 × CH_2), 5.45 (1H, s, ArH), 6.62 (1H, br s, NH) and 13.34 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.0, 22.5, 26.6, 26.7, 28.2, 30.0, 31.4, 32.8, 42.7, 47.5, 97.1, 150.5, 157.4, 176.4, 178.7 and 200.5.

4.17. 2,5-Bis-(naphthalen-2-ylamino- 3,6-dioxo-cyclohexa-1,4-dienecarboxylic acid methyl ester 18

4.17.1. Method F

Stirring time = 96 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:6; 1.5:5.5; 2:5) to afford a light-brown powder (0.0267 g, 20%). (**M+H**$^+$ Found: 449.1488. $C_{28}H_{20}N_2O_4$ requires M+H, 449.1501); R_f = 0.22 (EtOAc/hexane, 5:12). Mp = 201–203 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 2.97 (3H, s, CH_3), 6.07 (1H, s, ArH), 7.40–8.00 (14H, m, ArH), 8.39 (1H, br s, NH) and 8.70 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 51.4, 96.3, 120.3, 121.6, 122.0, 126.4, 126.6, 127.2, 127.6, 2 × 127.8, 129.4, 129.9, 133.2, 134.3, 146.5, 164.0 and 178.8.

4.18. 2,5-Bis[4-(2-hydroxyethyl)-phenylamino]-3,6-dioxo-cyclohexane-1,4-diene = carboxylic acid methyl ester 19

4.18.1. Method E

Stirring time = 72 h. Purification by flash chromatography (silica/Et_2O–hexane, 1:6; 1:3; 1:1) to afford a red solid (0.0205 g, 19%). (**M+H**$^+$ Found: 365.2460. $C_{20}H_{32}N_2O_4$ requires M+H, 365.2440); R_f = 0.27 (Et_2O/hexane, 1:1). Mp = 62–63 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.89 (6H, m, 2 × CH_3), 1.32 (10H, m, 5 × CH_2), 1.64 (6H, m, CH_2), 3.1.7 (4H, m, CH_2), 3.86 (3H, m, CH_3), 5.40 (1H, d, J = 7.8 Hz, NH) and 6.7 (1H, s, ArH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.0, 22.4, 22.5, 26.4, 26.6, 28.1, 29.0, 31.3, 42.7, 44.4, 51.8, 52.2, 92.7, 96.4, 147.9, 150.4, 166.9, 175.8, 176.2 and 178.1.

4.19. 2,5-Bis-hexylamino]-3,6-dioxo-cyclohexa-1,4-dienecarboxylic acid ethylester 20

4.19.1. Method E

Stirring time = 72 h. Purification by flash chromatography (silica/Et_2O–hexane, 1:6; 1:4; 1:2) to afford a red solid (0.0138 g, 12%). (**M**−**H**$^+$ Found: 379.2591. $C_{21}H_{35}N_2O_4$ requires M−H, 379.2597); R_f = 0.20 (Et_2O/hexane, 1:2). Mp = 63–65 °C. δ = 0.85 (6H, m, 2 × CH_3), 1.18–1.49. (10H, m, 5 × CH_2), 1.51–1.75 (6H, m, 3 × CH_2), 3.15 (3H, m, CH_3), 4.32 (2H, q, J = 7.2 and 7.9 Hz), 5.37 (1H, s, ArH), 6.64 (1H, br s, NH) and 7.23 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 13.9, 14.2, 22.4, 22.5, 26.4, 26.5, 28.2, 29.1, 42.7, 61.3, 92.6, 147.6, 150.5, 166.6, 176.0 and 176.5.

4.20. 2,5-Bis-(4isopropyl)-phenylamino]-3,6-dioxo-cyclohexa-1,4-dienecarboxylic acid ethylester 21

4.20.1. Method G

Stirring time = 72 h. Purification by flash chromatography (silica/Et_2O–hexane, 1.5:12; 1:6; 1:4; 1:2) to afford a dark-brown powder (0.0289 g, 21%). (**M+H**$^+$ Found: 447.2299. $C_{27}H_{28}N_2O_4$ requires M+H, 433.2127); R_f = 0.18 (Et_2O/hexane, 1:2). Mp = 118–120 °C [lit.[28] 132–133 °C]. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.96 (3H, t J = 7.0 and 7.2, CH_3), 1.3–1.7 (12H, m, 4 × CH_3), 2.80–3.10 (2H, m, 2 × CH), 3.51 (2H, q, CH_2), 6.06 (1H, s, ArH), 7.05–7.35 (8H, m, ArH), 8.16 (1H, br s, NH) and 8.65 (1H, s, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 13.8, 23.9, 33.8, 33,9, 60.8, 95.6, 123.1, 123.8, 127.2, 127.6, 134.4, 135.0, 147.0, 147.5, 148.1 and 178.6.

4.20.2. Method I

Stirring time = 72 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:10, 1:9, 1:8; 1:7; 1:6) to afford a brown-black powder (0.0390 g, 30%). R_f = 0.20 (EtOAc/hexane, 1:6).

4.21. 3-Acetyl-2,5-bis(2-pyidin-2-yl-ethylamino)-[1,4]benzoquinone 22

4.21.1. Method B

Stirring time = 92 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:2; EtOAc; MeOH/EtOAc, 1:19) to afford a brown solid (0.0235 g, 20%). (**M+H**$^+$ Found: 391.1789. $C_{22}H_{22}N_4O_3$ requires M+H 391.1770); R_f = 0.5 (MeOH/$CHCl_3$). Mp = 82–84 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 2.55 (3H, s, CH_3), 3.15 (4H, m, 2 × CH_2), 3.59 (2H, q J = 6.2 and 6.4 Hz), 3.42 (2H, q J = 6.2, 6.4 and 6.8 Hz), 5.52 (1H, s, ArH), 7.12–7.28 (4H, m, ArH), 7.63 (2H, t J = 7.6 Hz, ArH), 8.58 (2H, s, ArH) and 13.36 (1H, s, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 32.8, 35.9, 38.3, 41.7, 46.6, 97.1, 106.3, 121.7, 121.9, 123.2, 123.4, 136.5, 136.7, 149.5, 149.6, 150.6, 157.7, 158.2, 176.7, 178.2 and 200.3.

4.22. 3,6-Dioxo-2,5-bis-(2-pyridin-2-yl-ethylamino)-cyclohexa-1,4-dienecarboxylic acid ethyl ester 23

4.22.1. Method B

Stirring time = 96 h. Purification by flash chromatography (silica/EtOAc–hexane, 1:2; EtOAc; MeOH/EtOAc, 1:19) to afford a red–brown solid (0.0403 g, 32%). (**M+H**$^+$ Found: 421.1858. $C_{23}H_{24}N_4O_4$ requires M+H 421.1876); R_f = 0.20). Mp = 90–92 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.33 (3H, t J = 7.2 Hz, CH_3), 1.78 (1H, s, NH), 3.07 (4H, t J = 6.4 and 6.8 Hz, 2 × CH_2), 3.59 (4H, q J = 6.4 and 6.6 Hz, 2 × CH_2), 4.25 (2H, m, CH_2), 5.40 (1H, s, ArH), 7.14 (4H, t J = 5.2 and 7.6 Hz, ArH), 7.54–7.66 (2H, m, ArH), 8.00 (1H, s, NH) and 8.56 (2H, d J = 4.2 Hz, ArH); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.2, 30.9, 35.8, 36.4, 41.7, 43.4, 61.3, 92.8, 121.8, 123.1, 123.2, 136.6, 2 × 138.2, 149.5, 149.6, 150.2, 157.7, 176.0 and 176.6.

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