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Catecholamine-Protein Conjugates: Isolation of 4-Phenylphenoxazin-2-ones from Oxidative Coupling of N-Acetyldopamine with Aliphatic Amino Acids

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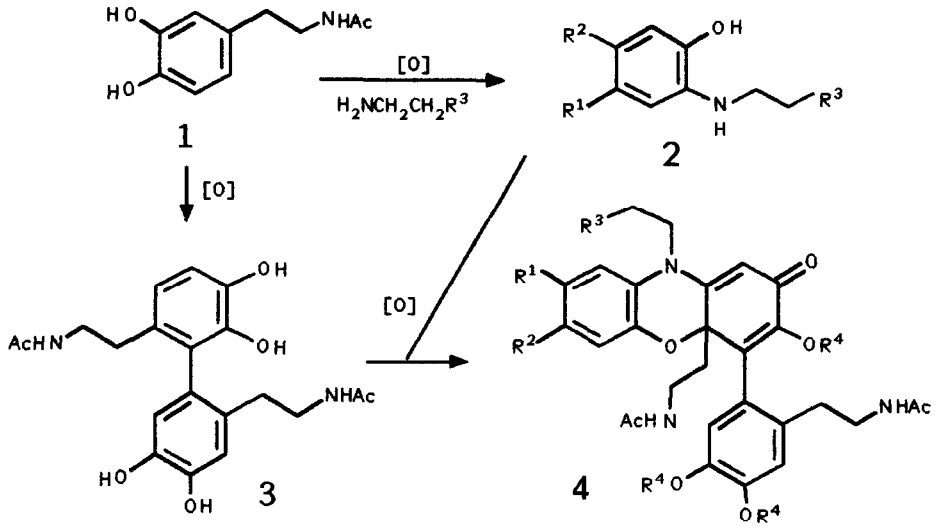
Abstract - 4-Phenylphenoxazinones **4** were isolated after biomimetic oxidation, using diphenoloxidases of insect cuticle, mushroom tyrosinase, or after autoxidation of N-acetyldopamine (**1**) in the presence of ß-alanine, ß-alanine methyl ester or N-acetyl-L-lysine. They are formed presumably by addition of 2-aminoalkyl-5-alkylphenols **2** to the *o*-quinone of biphenyltetrol **3** which, in turn, arises from oxidative coupling of **1**. The structures of **4** present the first examples for the assembly of reasonably stable intermediates in the rather complex process of chemical modifications of aliphatic amino acid residues by *o*-quinones.

Enzymatic oxidation of 1,2-diphenols is a widespread reaction in nature. It occurs commonly in the biosynthesis of polyphenolic biopolymers such as melanins, sclerotins, or phenolic adhesives. Intermediates are semiquinone radicals, *o*-quinones or *p*-quinone methides, which may cause allergenic, neurotoxic or general cytotoxic actions when they are generated at the wrong time in the wrong place. The beneficial as well as the deleterious effects of catechol oxidation in biological systems are ultimately caused by chemical modifications of proteins.[1] Though the molecular events leading to functional group modification in proteins seem reasonably clear on the basis of general reactivity of quinonoids, structures of reaction products have been identified only in very few instances. In an effort to fill this gap, we have recently isolated addition products of N-acetylhistidine to the *o*-quinone[2] as well as to the *p*-quinone methide[3] of N-acetyldopamine (**1**) which is one of the principal sclerotization agents of insect cuticle.[4,5] We now report on the structures of 4-phenylphenoxazinones **4** which are formed biomimeti-

cally by an unexpected oxidative trimerization of **1** in the presence of aliphatic amino acids. The isolation of the novel compounds **4** contributes a surprising detail in the very complex mosaic of polyphenolic natural products chemistry.

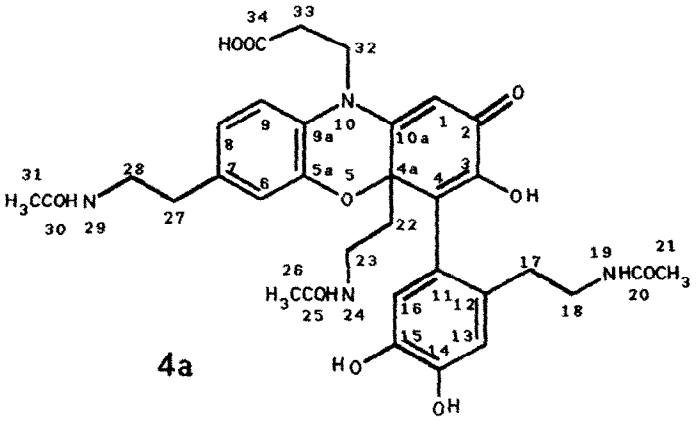


	R^1	R^2	R^3	R^4
4a:	H	CH_2CH_2NHAc	COOH	H
4b:	CH_2CH_2NHAc	H	COOH	H
4c:	H	CH_2CH_2NHAc	$COOCH_3$	H
4d:	H	CH_2CH_2NHAc	COOH	Ac
4e:	H	CH_2CH_2NHAc	$COOCH_3$	Ac
4f:	CH_2CH_2NHAc	H	$COOCH_3$	Ac
4g:	H	CH_2CH_2NHAc	$CH_2CH_2CH(NHAc)COOH$	H
4h:	CH_2CH_2NHAc	H	$CH_2CH_2CH(NHAc)COOH$	H

Incubation of a mixture of N-acetyldopamine and β-alanine in phosphate buffer with larval cuticle of the giant silkmoth *Hyalophora cecropia*[6] leads, within 6 h, to red-brown solutions. Chromatographic separation of the reaction mixture on a reversed phase C_{18} column affords two closely eluting yellow compounds. Both show virtually identical UV (λ_{max} = 265, 276, and 375 nm). PD-MS spectra (*m/z* = 651.5 [M+H⁺], and 565.8 [M+H⁺-CH_2CH_2NHAc]) acccount for a molecular formula $C_{33}H_{38}N_4O_{10}$, indicating with incorporation of one mole of β-alanine into a trimer of N-acetyldopamine with loss of one mole of H_2O.

NMR spectroscopy, including HH-COSY, HMQC[7] and HMBC[8] experiments (for review, see[9]), leads to assignment of structures **4a** and **4b**, respectively. The ^{13}C NMR spectrum of **4a** reveals 33 carbon atoms, five of

which account for a ketone (δ = 179.96 ppm), a carboxylic acid (δ = 172.70 ppm), and three N-acetyl groups (δ = 169.27, 169.23, and 169.09 ppm). Two diphenolic carbons appear at δ = 145.03 and 143.34 ppm, while one quaternary carbon is detected at δ = 75.98 ppm. The aliphatic region in the ^{1}H NMR spectrum of **4a** contains signals of eight methylene groups. Six of those are located in three acetamidoethyl groups, as revealed by 500 MHz HH-COSY experiments. This is confirmed by HMBC signals of C-20, C-25, and C-30 with NH-protons H-19, H-24, and H-29, and with methylene protons H-18, H-23, and H-28, respectively (see table 1). Likewise, carboxyl C-34 shows HMBC signals to H-32 (δ ^{13}C = 41.93 ppm) and H-33 (δ ^{13}C = 31.8 ppm), indicating a β-alanine moiety.



4a

In the ^{1}H NMR spectrum of **4a**, the 1,3,4-trisubstituted aromatic ring system is apparent with one proton at 6.83 ppm showing *o*-coupling to δ ^{1}H = 7.18 ppm and *m*-coupling to δ ^{1}H = 6.53 ppm. The assignments are deduced from HMBC and HMBQ experiments which, through correlation of the signals at δ ^{13}C = 135.76 ppm with H-27 and H-28 and of δ ^{13}C = 126.81 ppm with H-33 prove attachment of one acetamidoethyl group to C-7 and of the β-alanine nitrogen to C-9a, respectively. The 1,2,4,5-tetrasubstituted aromatic ring, containing the two phenoxy carbons and the second acetamido group attached to C-12 (δ = 130.96 ppm) is also evident from heteronuclear correlation experiments.

The remaining six carbon atoms belong to a tetrasubstituted cyclohexadienone partial structure containing carbonyl C-2 and quaternary C-4a. HMBC signals of the latter with H-22 (δ = 1.99 and 1.78 ppm) prove the position of the third acetamido group. The phenoxazine structure follows from correlation signals of C-10a (δ = 153.28 ppm) with H-32, indicating attachment of the β-alanine nitrogen to the cyclohexadienone, and from correlation of C-4a with C-5a *via* the oxygen bridge. Further correlation is observed in the HMBC experiment of C-4 with H-16 which

Table 1: NMR (^{1}H: 500 MHz; ^{13}C: 125 MHz; d_6-DMSO) data of **4a** and of **4b**.

Carbon number	**4a** δ ^{13}C	**4a** δ ^{1}H	**4b** δ ^{13}C	**4b** δ ^{1}H
1	97.97	5.78 (s)	98.04	5.78 (s)
2	179.96		179.83	
3	145.69		145.47	
4	118.50		118.49	
4a	75.98		75.64	
5a	140.73		138.99	
6	117.51	6.53 (d)	116.88	6.57 (d)
7	135.76		123.62	6.72 (d)
8	122.88	6.83 (dd)	134.03	
9	114.29	7.18 (d)	114.52	7.12 (s)
9a	126.81		128.17	
10a	153.28		153.19	
11	123.10		122.88	
12	130.96		130.68	
13	116.44	6.74 (s)	116.27	6.72 (s)
14	145.03		144.84	
15	143.34		143.17	
16	117.40	6.51 (s)	117.21	6.52 (s)
17	32.90	2.58/2.48 (2m)	32.67	2.56/2.49 (2m)
18	40.37	3.17 (m)	40.10	3.12 (m)
20	169.23		169.11	
21	22.73	1.72 (s)	22.59	1.71 (s)
22	32.05	1.99/1.78 (ddd/m)	31.84	1.99/1.75 (ddd/m)
23	34.50	3.08/2.91 (2dddd)	34.30	3.06/2.92 (2dddd)
25	169.09		168.88	
26	22.64	1.75 (s)	22.45	1.77 (s)
27	34.37	2.55/2.54 (2m)	34.72	2.65 (t)
28	40.16	3.17 (m)	40.15	3.25 (m)
30	169.27		169.06	
31	22.73	1.72 (s)	22.59	1.70 (s)
32	41.93	4.20/4.01 (2ddd)	42.05	4.21/4.03 (2ddd)
33	31.80	2.61 (m)	32.16	2.56 (m)
34	172.70		172.83	

δ (ppm) of NH-protons: **4a**: 7.80 (t, J = 5.2 Hz, H-19), 7.73 (t, J = 5.6 Hz, H-29), 7.59 (t, J = 5.4 Hz, H-24); **4b**: 7.88 (t, J = 5.4 Hz, H-19), 7.83 (t, J = 6.0 Hz, H-29), 7.59 (t, J = 5.4 Hz, H-24). HH-coupling constants (Hz): **4a**: H-32: ^2J = 15.2, ^3J = 9.1, 8.9, 6.5, 6.1; H-23: ^2J = 13.1, ^3J = 10.8, 10.5, 5.5, 5.3, J_{NH} = 5.4; H-22: ^2J = 12.5, ^3J = 10.5, 5.3; H-9,8,6: J_o = 8.4, J_m = 1.9. - **4b**: H-32: ^2J = 14.9, ^3J = 9.1, 8.1, 6.6, 6.5; H-27: ^3J = 7.3; H-23: ^2J = 12.9, ^3J = 11.1, 10.6, 5.5, 5.2, J_{NH} = 5.4; H-22: ^2J = 12.3, ^3J = 10.1, 5.9; H-7,6: J_o = 8.5.

proves the position of the tetrasubstituted aromatic ring at the phenoxazine ring system.

The ^{1}HNMR spectrum of **4b** is complementary to that of **4a** (table 1). The *m*-coupling of the low field H-9 is not resolved. However, *o*-coupling is now observed for H-7 and H-6. The benzylic protons H-27 (δ = 2.68 ppm) of the acetamido group at C-8 are, in this case, isochronic.

Treatment of phenoxazinone **4a** with AcCl/MeOH (16:100) gives monomethylester **4c**. (PD-MS: m/z = 666.1, [M+H$^+$]). Acetylation of **4a** yields triacetate **4d** (FAB-MS: m/z = 777.3, [M+H$^+$]). The mass spectra of **4c** and **4d** show a characteristic loss of 86 mass units, accounting for cleavage of the acetamidoethyl group at C-4a.

4-Phenyloxazinones are also formed upon oxidation of N-acetyldopamine (**1**) by means of mushroom tyrosinase in the presence of β-alanine methyl ester. In that case, the products were characterized as triacetates **4e** and **4f**. The EI-mass spectra display a prominent cleavage of the phenoxazine ring system resulting in a biphenyl ion $C_{26}H_{26}N_2O_9$ (m/z = 510) and a N-alkylquinonimine fragment (m/z = 280). As before, the regioisomers are easily distinguished by the coupling patterns of the trisubstituted aromatic ring. Due to acetylation, H-13 and H-16 are shifted downfield, when compared with the ^{1}H NMR spectra of **4a** and **4b**, respectively. The mass spectra show minor peaks of a tetraacetate (m/z = 834) and of a penta-acetate (m/z = 876). We assume that the higher masses are generated by acetylation of the cyclohexadienone carbonyl oxygen, and formation of the iminium acetate salt.

Oxidative coupling of **1** in the presence of N-acetyl-L-lysine affords two yellow compounds. Their UV spectra are virtually identical with the UV spectra of **4a** and **4b**. PD-MS reveals peaks of [M+Na$^+$] at m/z = 773.1 and of [M+H$^+$] at m/z = 750.6. From these data and from the assignment of structures **4a** and **4b** to the ß-alanine coupling products, it is concluded that the compounds obtained from **1** and N-acetyl-L-lysine are the 4-phenylphenoxazinones **4g** and **4h**, respectively. 4-Phenylphenoxazines are detected chromatographically also when an aqueous solution of **1** and β-alanine is exposed to air for a prolonged time.

Obviously, N^{10}-alkyl-4-phenyl-2,4a-dihydro-10*H*-phenoxazin-2-ones **4** are formed by the sequence of reactions depicted in the formula scheme. The final step is similar to the well known D-A-D-A[10] preparation of N-alkyl-phenoxazin-2,3-diones from *o*-(N-alkylamino)-phenols and 1,2-diphenols (*cf.*[11]), and to the biosynthesis of ommochromes and actinomycins from 3-hydroxy-anthranilic acid or kynurenic acid, respectively. Though the biphenyltetrol **3** is not proven as an intermediate, its occurrence is evident from earlier studies on oxidative phenolic coupling reactions of

1.[12,13,14] To the best of our knowledge, the generation of 4-phenyl-phenoxazin-2-ones from three moles of the parent diphenol and one mole of an aliphatic amino acid is unprecedented.

The results of this study suggest that oxidative modification of proteins by quinones in biological systems can lead to arylation of primary amino groups concomitant with the formation of structures of considerable complexity. Evidently, the assembly of 4-phenylphenoxazine-2-ones represents only one of many other types of phenolic coupling products. It is immediately evident that these modifications will increase the hydrophobicity of a protein dramatically. Exactly this is required for the formation of *e.g.* sclerotins in insect cuticles, or of adhesives in marine organisms such as molluscs and barnacles. With respect to humans, the understanding of the molecular principles of allergenic and cytotoxic actions that are caused by catechols will be significantly deepened by the consideration of 4-phenyl-phenoxazin-2-ones as elements of the structural modifications of proteins.

EXPERIMENTAL

UV: Unicam SP 8000 or Shimadzu UV-160A. - Chromatography: TLC: silica gel 60 F 25 on alumina sheets (Merck); prep. TLC on glass plates. SC: Bio-Beads SM-16 (Bio-Rad); Sephadex LH-20 (Pharmacia). HPLC: instrumentation from LKB or from Waters; Sperisorb ODS 2 (LKB; 4X250 mm column); μ-Porasil (Waters; 4X31 cm column). - ^{1}H NMR (360 or 400 or 500 MHz) and ^{13}C NMR (100.6 or 125 MHz): Bruker WM 360 or WM 400 or AMX 500 spectrometer (internal standard: TMS or solvent). - EI-MS: AEI MS-50; direct inlet; ionization energy 70 eV. - PD-MS: BioIon 20 plasma desorption mass spectrometer; the sample was applied from a 0.1% trifluoroacetic acid solution to a nitrocellulose covered target;[15] 10^6 start events. - FAB-MS: Kratos Concept 1H, matrix: *m*-NBA.

Oxidation of N-acetyldopamine* (1) *by means of diphenoloxidase of insect cuticle in the presence of ß-alanine.
Cleaned cuticle (*ca.* 2g fresh weight) from late fifth instar larvae of the giant silkmoth *Hyalophora cecropia*[6] is added to a mixture of 1 (390 mg, 2.0 mmol) and β-alanine (356 mg, 4.0 mmol) in 200 ml 0.2 M sodium phosphate buffer pH 7.6 and the mixture is gently shaken for 6 h at 40°C and then acidified to pH 3 with formic acid. Decantation is followed by pumping the solution onto Bio-Beads SM-16 (1.6X15 cm column, equilibrated with 0.2 M acetic acid; elution with a linear gradient 0->70% EtOH in 0.2 M acetic acid within 12 h). Fractions showing a yellow colour are combined, evaporated, and the residue subjected to chromatography on Sephadex LH-20 (2.6X50 cm column; elution with 0.2 M acetic acid) to afford 4±1 mg of 4a,7-bis-(2-acetamidoethyl)-4-[2-(2-acetamidoethyl)-4,5-dihydroxyphenyl]-10-(2-carboxyethyl)-3-hydroxy-2,4a-dihydro-10*H*-phenoxazin-2-one (**4a**) (elution volume: 650 ml) and 4±1 mg (0.3%) of 4a,8-bis-(2-acetamidoethyl)-4-[2-(2-acetamidoethyl)-4,5-dihydroxyphenyl]-10-(2-carboxyethyl)-3-hydroxy-2,4a-dihydro-10*H*-phenoxazin-2-one (**4b**) (elution volume: 800 ml). The compounds are chromatographically >95% pure, as

revealed by HPLC on Spherisorb ODS 2 (elution with a linear gradient 0->70% MeOH in 0.1% trifluoroacetic acid; flow rate: 0.5 ml·min^{-1}).
4a: Yellow resin. - HPLC: t_R = 22.3 min. - UV (MeOH/H_2O): λ_{max} = 264, 276s, 373 nm. - ^1H and ^{13}C NMR: see table 1. - PD-MS: *m/z* = 674.3 [M+Na$^+$], 651.6 [M+H$^+$], 565.8 [M+H$^+$-CH_2CH_2NHAc], 302.0, 279.7.
4b: Yellow resin. - HPLC: t_R = 23.1 min. - UV (MeOH/H_2O): λ_{max} = 266s, 276, 378 nm. - ^1H and ^{13}C NMR: see table 1. - PD-MS: *m/z* = 674.3 [M+Na$^+$], 651.6 [M+H$^+$], 565.8 [M+H$^+$-CH_2CH_2NHAc].

Methyl ester **4c**: A small aliquot of **4a** is methylated by reaction with 2 N methanolic HCl, prepared by mixing AcCl/MeOH (16:100 v/v), for 2 h at 20°C, followed by evaporation of the reagent to afford 4a,7-bis-(2-acetamidoethyl)-4-[2-(2-acetamidoethyl)-4,5-dihydroxyphenyl]-10-(2-carbomethoxyethyl)-3-hydroxy-2,4a-dihydro-10*H*- phenoxazin-2-one (**4c**). - PD-MS: *m/z* = 689.9 [M+Na$^+$], 666.1 [M+H$^+$], 594.0 [M+H$^+$-CH_2NHAc], 579.8 [M+H$^+$-CH_2CH_2-NHAc].

Triacetate **4d**: From acetylation of 10.5 mg (16.2 µmol) **4a** in 0.8 ml Ac_2O and 20 µl pyridine. Prep. TLC ($CHCl_3$/MeOH = 2:1) affords 8.6 mg of 4a,7-bis-(2-acetamidoethyl)-4-[2-(2-acetamidoethyl)-4,5-diacetyloxyphenyl]-3-acetyloxy-10-(2-carboxyethyl)-2,4a-dihydro-10*H*-phenoxazin-2-one (**4d**). - Yellow resin. - UV (MeOH): λ_{max} (lg ϵ) = 267 (3.94), 400 nm (3.71). - FAB-MS: *m/z* = 799.3 [M+Na$^+$], 777.3 [M+H$^+$], 757.3 [M+Na$^+$-$C_2H_2O_2$], 691.3 [M+H$^+$-CH_2CH_2NHAc], 649.2 [M+H$^+$-$C_2H_2O_2$-CH_2CH_2NHAc], 607.2 [M+H$^+$-2 $C_2H_2O_2$-CH_2CH_2NHAc].

Oxidation of N-acetyldopamine* (1) *by means of mushroom tyrosinase in the presence of ß-alanine methyl ester.
A solution of 2.05 g (10.5 mmol) N-acetyldopamine and 146 mg (1.04 mmol) β-alanine methyl ester hydrochloride in 110 ml 10 mM potassium phosphate buffer pH 7.2 is stirred with 6 mg (8400 U) tyrosinase (Sigma) for 26 h at 20°C. Evaporation is followed by gel filtration of the residue on Sephadex LH-20 (2.2X15 cm column; elution with H_2O). Fraction I with peak center at 770 ml contains N-acetyldopamine (1.10 g; 53.5%). Fraction II (102 mg) and III (113 mg) with peak centers at 1620 and 2050 ml, respectively, contain two yellow components.[16] Fraction II: TLC ($CHCl_3$/12% MeOH): R_f = 0.33. - UV (MeOH): λ_{max} = 226, 268, 393 nm. - Acetylation of Fraction II is carried out by stirring the dry evaporation residue in a mixture of 1.0 ml pyridine and 0.5 ml acetic anhydride for 2 h at 20°C. Evaporation of the reaction mixture is followed by prep. TLC ($CHCl_3$/12% MeOH) and, subsequently, HPLC on µ-Porasil (elution with CH_2Cl_2/3.5 MeOH; flow rate: 1.0 ml·min^{-1}) to afford 13.8 mg of 4a,7-bis-(2-acetamidoethyl)-4-[2-(2-acetamidoethyl)-4,5-diacetyloxyphenyl]-3-acetyloxy-10-(2-carbomethoxyethyl)-2,4a-dihydro-10*H*-phenoxazin-2-one (**4e**) and 17.9 mg of 4a,8-bis-(2-acetamidoethyl)-4-[2-(2-acetamidoethyl)-4,5-diacetyloxyphenyl]-3-acetyloxy-10-(2-carbomethoxyethyl)-2,4a-dihydro-10*H*-phenoxazin-2-one (**4f**).
4e: - Yellow resin. - TLC ($CHCl_3$/12% MeOH): R_f = 0.47. - HPLC: t_r = 24.4 min. - UV (MeOH): λ_{max} = 222, 267, 394 nm. - ^1H NMR (360 MHz, $CDCl_3$): δ = 7.24 (s, H-13), 7.06 (s, H-16), 6.99 (d, J_o = 8.7 Hz, H-9), 6.84 (dd, J_o = 8.7 Hz, J_m = 1.8 Hz, H-8), 6.60 (d, J_m = 1.8 Hz, H-6), 6.34 (br.s, NH), 5.78 (s, H-1 and NH), 5.67 (br.t, NH), 4.23 (m, H_A-32), 4.11 (m, H_B-32), 3.74 (s, -OCH_3), 3.69, 3.49, 3.38 (3 m, 6 H, H-18,23,28), 3.15, 2.85, 2.79 (3 m, H-17,22,27), 2.69 (t, *J* = 7.5 Hz, H-33), 2.34, 2.29, 2.11, (3 s, 3 H_3COO-), 1.91, 1.90, 1.88 (3 s, 3 H_3COONH-). - EI-MS (70 eV): m/z = 834 (0.6) [M+2H$^+$] of tetra-acetate, 792 (0.8) [M+2H]$^+$, 510.1638 (6; calcd. for $C_{26}H_{26}N_2O_9$: 510.1649), 468.1533 (20, calcd. for $C_{24}H_{24}N_2O_8$: 468.1535), 426 (16), 413 (8), 384 (7), 371 (7), 367 (6), 325 (6), 310 (5), 280 (5), 270 (6), 259 (5), 221 (10), 219 (11), 208 (7), 178 (8), 161 (6), 159 (10), 148 (8), 137 (8), 136.0524 (59, calc for $C_8H_8O_2$:

136.0522), 135 (14), 134 (6), 123 (11), 116 (9), 85 (20), 84 (7), 78 (7), 77 (6), 74 (15), 72 (7), 69 (11), 60 (43), 59 (32), 58 (6), 57 (7), 55 (51), 45 (41), 44 (48), 43 (72), 42 (100), 41 (70), 40 (16), 32 (85), 31 (95), 29 (59), 28 (82).

4f: Yellow resin. - TLC ($CHCl_3$/12% MeOH): R_f = 0.47. - HPLC: t_r = 31.9 min. - UV (MeOH): λ_{max} = 222, 267, 391 nm. - IR ($CHCl_3$): ν = 3500*w*, 3030*w*,1785*s*, 1775*s*, 1670*s*, 1575*s*, 1373*s*. - ^{1}H NMR (360 MHz, $CDCl_3$): δ = 7.23 (s, H-13), 7.04 (s, H-16), 6.91 (d, J_m = 1.5 Hz, H-9), 6.76 (dd, J_o = 8.5 Hz, J_m = 1.5 Hz, H-7), 6.65 (d, J_o = 8.5 Hz, H-6), 6.35 (br., NH), 5.78 (s, H-1 and NH), 5.61 (br.t, NH), 4.24 (m, H_A-32), 4.13 (m, H_B-32), 3.73 (s, $-OCH_3$), 3.49-3.36 (3 m, H-18,23,28), 3.2-2.7 (m, with t at 2.86, J = 7.5 Hz, and q at 2.78, J = 7.5 Hz, H-18,23,28,33), 2.34, 2.29, 2.11 (3 s, 3 H_3CCOO-), 1.96, 1.90, 1.87 (3 s, 3 H_3CCONH-). - EI-MS (70 eV): *m/z* = 876 (1, [M+2H$^+$] of penta-acetate), 834 (5, [M+2H$^+$] of tetra-acetate), 792.3218 (9, [M+2H]$^+$, calc. for $C_{40}H_{48}N_4O_{13}$: 792.3186), 750 (6), 691 (6), 510 (4), 468 (16), 426 (17), 413 (13), 384 (14), 367 (7), 325 (15), 310 (11), 282 (6), 280 (6), 270 (10), 268 (12), 255 (6), 252 (8), 221 (14), 178 (6), 159 (16), 148 (6), 147 (7), 146 (10), 136 (15), 135 (16), 134 (6), 122 (7), 116 (17), 88 (10), 85 (40), 84 (10), 75 (47), 60 (40), 59 (33), 58 (18), 55 (64), 45 (44), 44 (44), 43 (89), 42 (100), 41 (36), 32 (76), 31 (95), 30 (27), 29 (29), 28 (80).

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