Synthesis of APTRA Derivatives as Building Blocks for Low-Affinity Fluorescent Ca^{2+} Indicators

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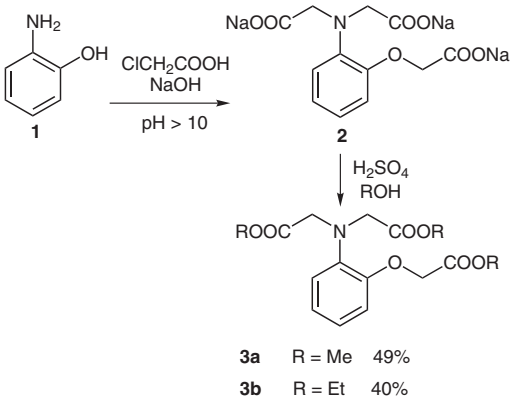
Abstract: A new method for the synthesis of trialkyl aminophenoltriacetates (APTRA triesters) and the functionalization of these into suitable building blocks for potential fully conjugated low-affinity fluorescent Ca^{2+} indicators are described.

Key words: heterocycles, supramolecular chemistry, aminophenoltriacetate, intracellular calcium, fluorescence, indicator

From a functional point of view, Ca^{2+} is probably the most important intracellular cation. Measurements of (changes in) cytosolic free Ca^{2+} concentrations with fluorescent probes have enabled scientists to investigate the role of Ca^{2+} in numerous physiological processes. The development of fluorescent indicators, with APTRA[1] (*o*-aminophenol-*N*,*N*,*O*-triacetic acid) and BAPTA [1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid][2] moieties as Ca^{2+} chelators, showing shifts in their excitation and/or emission spectra upon binding Ca^{2+}, entailed a major breakthrough in the elucidation of intracellular Ca^{2+} dynamics.[3] Cells at rest have a cytosolic free Ca^{2+} concentration of around 100 nM; upon activation of the cells, this level can rise to well above 1 μM. These changes play a pivotal role in many cellular processes, ranging from muscle contraction, neuronal signaling, secretion, fertilization, cell division, to metabolism and cell death.[4] New fluorescent Ca^{2+} indicators with improved properties can supplement the list of commercially available Ca^{2+} indicators.[5] Therefore, in this respect, we designed and synthesized a number of new APTRA derivatives allowing coupling with previously used fluorophores as well as direct transformation into more novel conjugates.

To prepare the APTRA-based fluorescent Ca^{2+} indicators we needed to put the appropriate substituents on the APTRA moiety. We also looked for an easier and cheaper method to synthesize the APTRA triester **3** on a large scale (10–100 g). For the reported synthesis, expensive bases (e.g. proton sponge) and purification by column chromatography were necessary.[7] Yields were suppressed due to the formation of benzoxazinone side-products after cyclization of the intermediates.

The new method for synthesizing APTRA consists of an adaptation of a known reaction[6] between *o*-aminophenol (**1**) and chloroacetic acid in water while keeping the pH between 10 and 12 by addition of NaOH (Scheme 1). The yield of **3** was found to drop when an equivalent amount of NaOH was added in one portion. Because of the lack of nucleophilicity of the carboxylate anions, cyclization was avoided. Subsequent evaporation of water and Fischer esterification of **2** (using 1.1 equiv of H_2SO_4 with respect to the amount of NaOH used in the previous step) gave the triester of APTRA in fair yields and good purity. Attempts to prepare the tribenzyl APTRA by Fischer esterification of APTRA **2** with benzyl alcohol or benzylation in basic media with benzyl bromide failed. The prepared trialkyl *o*-aminophenol-*N*,*N*,O-triacetates **3a** and **3b** were used as substrates for the synthesis of APTRA building blocks.



Scheme 1

The synthesis of a previously described[7] derivative, the 5-bromo APTRA triester **4**, was optimized. The use of bromine and CH_2Cl_2 as solvent at room temperature instead of NBS in AcOH reduced the reaction time and gave a higher yield (Scheme 2). Several attempts to iodinate **3** always gave small amounts of persubstituted side-products that were difficult to remove.

To prepare the 5-pinacolatoboronic ester derivative **5** starting from **4** (Scheme 2), it was necessary to use microwave irradiation because of dehalogenation occurring under conventional conditions. To this end we adapted our published conditions for the microwave assisted synthesis of electron-rich aromatic boronic esters.[8] Both **4** and **5** have potential as reagents for coupling with other arenes in order to extend the conjugated system. This could result in APTRA derivatives that show fluorescence at an appropriate wavelength.

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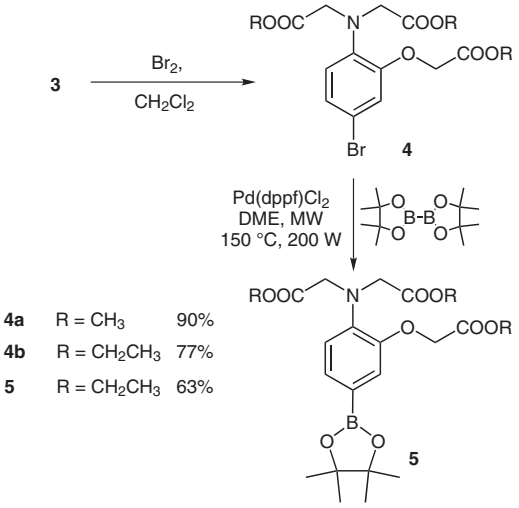
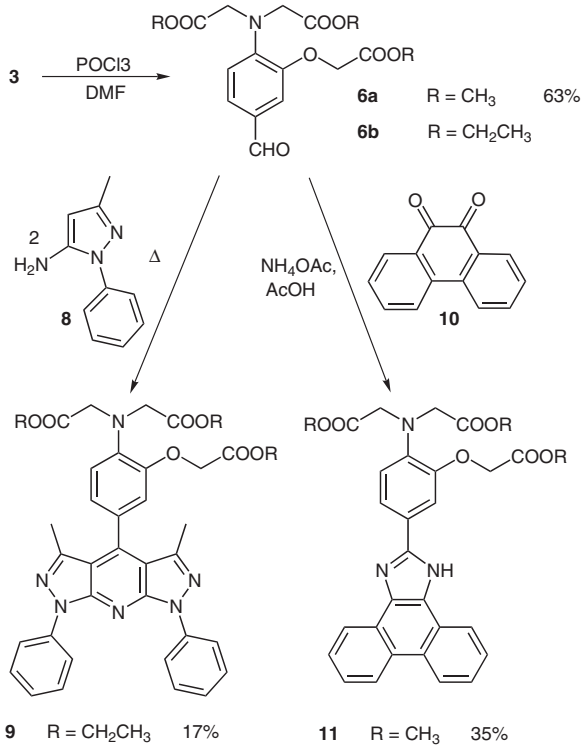


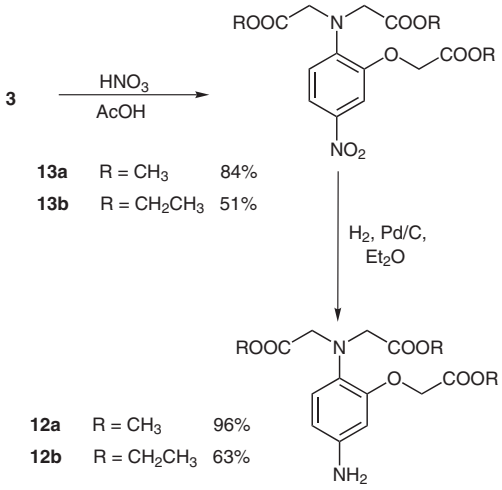
Scheme 2

Formylation of **3a**,**b** under Vilsmeier–Haack conditions gave 5-formyl APTRA triesters **6a**,**b** in acceptable yields (Scheme 3).

The [2+1]-condensation reaction of aldehyde **6b** with 5-amino-3-methyl-1-phenylpyrazole (**8**) gave tricyclic compound **9** in low yield (Scheme 3). The low yield may be due to intramolecular Claisen-type condensation between the acetate functions. Analogs of **9** were described by us to be highly fluorescent.[9] Condensation of aldehyde **6a** with 9,10-phenanthrenequinone (**10**) in the presence of ammonium acetate gave **11** in moderate yield (Scheme 3).



Scheme 3



Scheme 4

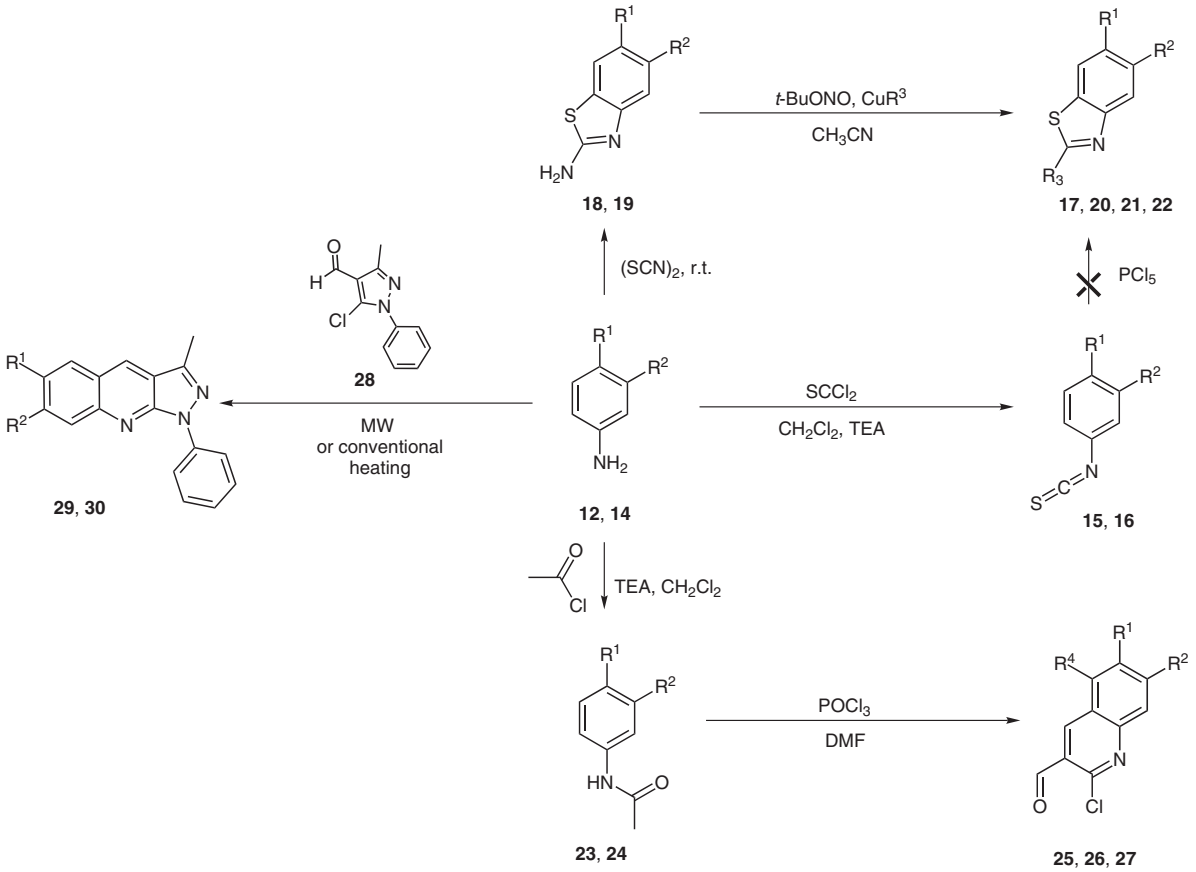
The very useful 4-amino-APTRA triesters **12** were prepared by subsequent nitration of **3** to **13** and reduction of **13** to **12** (Scheme 4). These products constitute the key intermediates in the synthesis of fused APTRA analogues (Scheme 5).

As a model compound for the APTRA triester **12** we have used the commercial 4-aminoveratrol (**14**) to tune the reaction conditions leading to benzothiazole and quinoline derivatives (Table 1).

Model compound **14** and 4-amino-APTRA triester **12b** were easily transformed into isothiocyanates **15** and **16** with thiophosgene. An earlier attempt to prepare 2-chlorobenzothiazole **17** from 4-isothiocyanatoveratrol with PCl_5 had failed,[10] probably due to the sensitivity of the electron-rich aromatic nucleus to the harsh reaction conditions. As an alternative, we prepared the 2-aminobenzothiazole derivatives **18** and **19** by reaction of the anilines **12** or **14** with freshly prepared thiocyanogen[11] followed by Sandmeyer reaction under anhydrous conditions to afford the 2-halobenzothiazoles **17**, **20**, **21** and **22**.

Quinolines can be prepared from the acetanilides **23** and **24** by a Vilsmeier-type methodology reported by Meth–Cohn.[12] The reaction with model compound **23** gave the expected quinoline **25** in good yield. Unfortunately, starting from anilide **24** and 8 equivalents of DMF, beside the expected quinoline **26**, substantial amounts of diformylated product **27** were found. Optimal results were obtained using 2 equivalents of DMF, affording **26** in moderate yield. The halogen substituents on the benzothiazole and quinoline are easily substituted with aryl groups using the Suzuki[13] or Stille[14] coupling reactions. We are currently investigating the scope of these coupling reactions.

Condensation reactions of anilines with *o*-chloroaldehydes can give fully conjugated aromatic heterocycles. The solventless condensation reaction at 190–200 °C of 4-aminoveratrol (**14**) and 5-chloro-4-formyl-3-methyl-1-phenylpyrazole (**28**) gave the expected pyrazolo[3,4-*b*]quinoline **29** in acceptable yield (Scheme 5). An APTRA triester functionalized pyrazolo[3,4-*b*]quinoline



Scheme 5

30 was prepared in low yield by heating (conventional or microwave assisted) an equimolar mixture of **12a** and **28** without solvent. The low yield may again be due to intramolecular Claisen-type condensation between the acetate functions.

In conclusion, we have demonstrated that functionalization of APTRA triesters **3** can give suitable building blocks **4**, **5**, **6**, **12**, **16**, **21**, **22**, and **26**. These compounds offer numerous possibilities for further derivatization by condensation reactions or by transition metal catalyzed reactions into custom-made fluorescent systems which, upon saponification, can serve as low-affinity fluorescent Ca^{2+}indicators.

The fluorescence data of **9**, **11** and **30** and their use as a Ca^{2+}indicators is under investigation and will be reported elsewhere.

DMF, THF and Et_2O were dried over molecular sieve 4 Å. All other reagents and solvents were purchased from Acros Organics or Aldrich and used without further purification. Mass spectra were recorded on a Kratos MSTC instrument at 70 eV or Hewlett Pacard 5989A Quadrupole. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 300 MHz.

Trisodium *o*-Aminophenol-*N*,*N*,O-triacetate APTRA (2)

To a three-necked flask equipped with a pH meter and a reflux condenser, charged with *o*-aminophenol (**1**, 10.9 g, 0.10 mol) and chloroacetic acid (47.2 g, 0.50 mol), was added an aqueous solution of NaOH (28.0 g, 0.70 mol in 100 mL H_2O). The mixture was heated in a preheated oil bath to 100 °C. Solid NaOH was added portionwise when the pH dropped below 10. After the pH remained constant around 10.7 the mixture was refluxed for another 30 min. After cooling, the water was evaporated under reduced pressure. The crude mixture of **2**, excess NaOH and acetate residues was used in further synthesis without purification.

^{1}H NMR (300 MHz, D_2O): δ = 3.74 (s, 4 H, NCH_2), 4.60 (s, 2 H, OCH_2), 7.03 (d, *J* = 8.1 Hz, 1 H, C_6H_4), 7.10 (t, *J* = 7.3 Hz, 1 H, C_6H_4), 7.21 (t, *J* = 7.3 Hz, 1 H, C_6H_4), 7.28 (d, *J* = 8.1 Hz, 1 H, C_6H_4).

^{13}C NMR (75 MHz, D_2O): δ = 180.1, 177.3, 150.4, 139.2, 124.2, 122.6, 120.5, 113.5, 67.2, 58.0.

Triethyl *o*-Aminophenol-*N*,*N*,*O*-triacetate (3b)

To the crude residue obtained in the preparation of **2** were added EtOH (350 mL) and H_2SO_4 (75.0 g, 40.8 mL, 0.77 mol) and the mixture was stirred for 3 d at reflux temperature. After cooling the salts were filtered off and the solvent was evaporated. The residue was dissolved in EtOAc and washed with a 10% NaOH solution and then with brine. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure yielding compound **3b** (15.8 g, 43% calculated from *o*-aminophenol) as a pale brown oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.22 (t, *J* = 7.3 Hz, 6 H, OCH_3), 1.30 (t, *J* = 7.3 Hz, 3 H, OCH_3), 4.15 (q, *J* = 7.1 Hz, 4 H, OCH_2), 4.20 (s, 4 H, NCH_2), 4.24 (q, *J* = 7.1 Hz, 2 H, OCH_2), 4.65 (s, 2 H, OCH_2), 6.80–6.95 (m, 4 H, C_6H_4).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.2, 169.3, 150.4, 140.2, 123.4, 122.2, 120.8, 115.2, 66.6, 61.4, 54.3, 14.7, 14.3.

LR–MS (CI): *m*/*z* = 368 [MH^+].

Table 1 Synthesis and Use of Key Intermediate 4-Amino APTRA Triester **12** and Its Model Compound 4-Aminoveratrol (**14**)

Product	R^1	R^2	R^3	R^4	Yield (%)
15	OMe	OMe			93
16	$N(CH_2COOEt)_2$	OCH_2COOEt			83
17	OMe	OMe	Cl		48
18	OMe	OMe			72[a]
19a	$N(CH_2COOMe)_2$	OCH_2COOMe			55
19b	$N(CH_2COOEt)_2$	OCH_2COOEt			35
20	OMe	OMe	Br		68
21	$N(CH_2COOMe)_2$	OCH_2COOMe	Cl		28
22	$N(CH_2COOMe)_2$	OCH_2COOMe	Br		66
23	OMe	OMe			47
24	$N(CH_2COOMe)_2$	OCH_2COOMe			n.d.
25	OMe	OMe		H	56
26	$N(CH_2COOMe)_2$	OCH_2COOMe		H	15,[b] 33[c]
27	$N(CH_2COOMe)_2$	OCH_2COOMe		CHO	22,[b] trace[c]
29	OMe	OMe			52
30	$N(CH_2COOMe)_2$	OCH_2COOMe			20

[a] In a one-pot reaction: **14**, KSCN, Br_2 in MeOH.
[b] Using 8 equiv DMF.
[c] Using 2 equiv DMF.

HR–MS (EI): *m*/*z* calcd for $C_{18}H_{25}NO_7$: 367.1631; found: 367.1632.

Trimethyl *o*-Aminophenol-*N*,*N*,*O*-triacetate (3a)
Compound **3a** was prepared in the same way as **3b**. Yield: 15.8 g (48.4% starting from *o*-aminophenol); white crystals after crystallisation from diisopropyl ether; mp 65 °C (Lit.[7] 75 °C).

^{1}H NMR and ^{13}C NMR spectra were identical to the previously reported ones.[7]

4-Bromo-2-ethoxycarbonylmethoxy-*N*,*N*-bis(ethoxycarbonylmethyl)aniline (4b)
A solution of **3b** (1.84 g, 5.00 mmol) in CH_2Cl_2 (80 mL) was cooled to 0 °C in an ice bath. Br_2 (880 mg, 282 μL 5.50 mmol) dissolved in CH_2Cl_2 (10 mL) was added in a dropwise manner to the mixture. After 2 h stirring at r.t., the reaction mixture was quenched with an aq NaOH solution (10%). The organic layer was washed with water and then dried over $MgSO_4$. After evaporation of the solvent, **4b** (1.72 g, 77%) was obtained as a pale brown oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.16–1.26 (m, 9 H, OCH_3), 4.10–4.19 (m, 8 H, NCH_2, OCH_2), 4.26 (q, *J* = 7.1 Hz, 2 H, OCH_2), 4.60 (s, 2 H, OCH_2), 6.73 (d, *J* = 8.8 Hz, 1 H, C_6H_3), 6.87 (d, *J* = 2.2 Hz, 1 H, C_6H_3), 7.05 (dd, *J* = 8.8, 2.2 Hz, 1 H, C_6H_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.4, 168.9, 150.6, 140.0, 131.9, 124.5, 121.9, 84.2, 66.6, 61.6, 61.1, 53.9, 43.2, 14.6, 14.4.

LR–MS (CI): *m*/*z* = 446–448 [MH^+].

HR–MS (EI): *m*/*z* calcd for $C_{18}H_{24}BrNO_7$: 445.0736; found: 445.0726.

4-Bromo-2-methoxycarbonylmethoxy-*N*,*N*-bis(methoxycarbonylmethyl)aniline (4a)
Compound **4a** was prepared by the same procedure as **4b** giving **4a** (3.92 g, 97%) as white crystals; mp 72–73 °C (Lit.[7] 58–60 °C).

^{1}H NMR and ^{13}C NMR spectra were identical to those previously reported.[7]

2-Ethoxycarbonylmethoxy-*N*,*N*-bis(ethoxycarbonylmethyl)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline (5)
A solution of **4b** (530 mg, 1.20 mmol), bis(pinacolato)diboron (329 mg, 1.30 mmol), KOAc (294 mg, 3.00 mmol) and Pd[1,1′-bis(diphenylphosphino)ferrocene]Cl_2 (30 mg, 3 mol%) in ethylene glycol dimethyl ether (DME, 2 mL) was heated in a microwave oven to 150 °C at a power of 200 W. After 15 min the solution was cooled and filtered through a layer of Celite. The Celite was washed with Et_2O. After removing of the solvent, the residue was purified by column chromatography on silica gel (eluent: CH_2Cl_2–EtOAc, 20:1). Compound **5** (370 mg, 63%) was obtained as a pale brown viscous oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.22–1.27 (m, 9 H, OCH_3), 1.30 [s, 12 H, $C(CH_3)_3$], 4.14–4.26 (m, 10 H, OCH_2, NCH_2), 4.66 (s, 2 H, OCH_2), 6.82 (d, *J* = 8.1 Hz, 1 H, C_6H_3), 7.20 (d, *J* = 1.5 Hz, 1 H, C_6H_3), 7.38 (d, *J* = 8.1, 1.5 Hz, 1 H, C_6H_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.5, 169.2, 148.9, 142.6, 130.0, 120.5, 118.7, 83.9, 66.0, 61.4, 61.0, 54.1, 25.2, 14.5.

LR–MS (CI): *m*/*z* = 494 [MH^+].

HR–MS (EI): *m/z* calcd for $C_{24}H_{36}BNO_9$: 493.2483; found: 493.2488.

4-Formyl-2-ethoxycarbonylmethoxy-*N*,*N*-bis(ethoxycarbonylmethyl)aniline (6b)

To a solution of **3b** (1.47 g, 4.00 mmol), pyridine (388 mg, 0.40 mL, 5.00 mmol) in DMF (3.76 g, 4,0 mL, 50.0 mmol) was added $POCl_3$ (4.94 g, 3.00 mL, 32.0 mmol) in a dropwise manner at 0 °C over 5–10 min. The reaction was heated for 1–2 h at 65 °C until completion. The reaction was monitored by the disappearance of the starting material on silica TLC plate. After completion of the reaction, the reaction mixture was poured into ice-water . After extraction with EtOAc the organic layer was washed with brine and dried over $MgSO_4$. After removal of the solvent under reduced pressure, the residue was purified by column chromatography on silica gel (eluent: CH_2Cl_2–EtOAc, 9:1). This afforded **6b** (1.33 g, 61%) as white crystals; mp 59–60 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.25–1.31 (m, 9 H, OCH_3), 4.21–4.27 (m, 10 H, NCH_2, OCH_2), 4.65 (s, 2 H, OCH_2), 6.80 (d, *J* = 8.1 Hz, 1 H, C_6H_3), 7.27 (d, *J* = 1.8 Hz, 1 H, C_6H_3), 7.44 (dd, *J* = 8.1, 1.8 Hz, 1 H, C_6H_3,), 9.76 (s, 1 H, CHO).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 190.3, 170.7, 168.0, 148.7, 145.3, 129.8, 127.3, 117.2, 111.6, 65.6, 61.4, 61.0, 54.0, 14.2, 14.1.

LR–MS (CI): *m/z* = 396 [MH^+].

HR–MS (EI): *m/z* calcd for $C_{19}H_{25}NO_8$: 395.1580; found: 395.1574.

4-Formyl-2-methoxycarbonylmethoxy-*N*,*N*-bis(methoxycarbonylmethyl)aniline (6a)

Compound **6a** was prepared in the same way as **6b** giving **6a** (4.24 g, 60%) as white crystals; mp 94 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.78 (s, 6 H, OCH_3), 3.80 (s, 3 H, OCH_3), 4.29 (s, 4 H, NCH_2), 4.67 (s, 2 H, OCH_2), 6.80 (d, *J* = 8.8 Hz, 1 H, C_6H_3), 7.27 (d, *J* = 1.5 Hz, 1 H, C_6H_3), 7.43 (dd, *J* = 8.8, 1.5 Hz, 1 H, C_6H_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 190.7, 171.5, 168.5, 148.9, 145.5, 130.3, 127.8 (CH), 117.5 (CH), 111.9, 65.8, 54.2, 52.6, 52.4.

LR–MS (CI): *m/z* = 354 [MH^+].

HR–MS (EI): *m/z* calcd for $C_{16}H_{19}NO_8$: 353.1111; found: 353.1111.

4-(3,5-Dimethyl-1,7-diphenyl-1,7-dihydrodipyrazolo[3,4-*b*:4,3-*e*]-2-ethoxycarbonylmethoxy-*N*,*N*-bis(ethoxycarbonylmethyl)aniline (9)

Compound **6b** (99 mg, 0.25 mmol) and 5-amino-1-phenyl-3-methylpyrazole (**8**, 108 mg, 0.75 mmol) were heated in a long tube under Ar to 120–160 °C for 1 h and 2 min at 200 °C. After cooling, EtOH (8 mL) was added and the mixture was heated at reflux temperature for 20 min. The solvent was evaporated and the obtained residue was purified by column chromatography on silica gel (eluent: CH_2Cl_2 to CH_2Cl_2–EtOAc, 80:20) to give **9** (30 mg, 17%) as yellow glass film; mp 141 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.20–1.30 (m, 9 H, OCH_3), 2.10 (s, 6 H, pyrazole-CH_3), 4.21–4.42 (m, 10 H, OCH_2, NCH_2), 4.67 (s, 2 H, OCH_2), 6.87 (s, 1 H, C_6H_3), 6.99–7.05 (m, 2 H, C_6H_3), 7.31 (t, *J* = 8.1 Hz, 2 H, C_6H_5), 7.52 (t, *J* = 8.1 Hz, 4 H, C_6H_5), 7.52 (d, *J* = 8.1 Hz, 4 H, C_6H_5).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.6, 168.7, 150.9, 149.2, 144.9, 141.4, 140.8, 129.3, 127.9, 125.5, 123.4, 120.6, 119.2, 115.1, 114.0, 66.3, 61.7, 61.3, 54.2, 15.3, 14.6, 14.5.

LR–MS (CI): *m/z* = 705 [MH^+].

HR–MS (EI): *m/z* calcd for $C_{39}H_{40}N_6O_7$: 704.2958; found: 704.2971.

2-Methoxycarbonylmethoxy-*N*,*N*-bis(methoxycarbonylmethyl)-4-(1*H*-phenathro[9,10-*d*]-imidazol-2yl)aniline (11)

A solution of **6a** (177 mg, 0.50 mmol), 9,10-phenanthrenequinone (**10**, 104 mg, 0.50 mmol) and ammoniumacetate (385 mg, 5.00 mmol) in glacial AcOH was charged in a 10 mL glass tube which was then tightly sealed with an aluminum/Teflon crimp and heated at 180 °C by irradiation at 250 W for 2 min in a CEM-Discover mono-mode microwave apparatus. The cooled solid mixture was poured in crushed ice and then neutralized with an aq NH_3 solution (10%). The precipitate was filtered and purified by column chromatography on silica gel (eluent: CH_2Cl_2 to CH_2Cl_2–EtOAc, 90:10). Compound **11** (95 mg, 35%) was obtained as a white-yellow powder; mp 199–200 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.66 (s, 6 H, OCH_3), 3.77 (s, 3 H, OCH_3), 4.29 (s, 4 H, NCH_2), 4.93 (s, 2 H, OCH_2), 6.95 (s, *J* = 8.1 Hz, 1 H, C_6H_3), 7.63 (t, *J* = 7.3 Hz, 2 H), 7.75 (t, *J* = 7.3 Hz, 2 H), 7.77 (s, 1 H, C_6H_3), 7.83 (d, *J* = 8.8 Hz, 1 H, C_6H_3), 8.54 (d, *J* = 8.1 Hz, 2 H), 8.85 (d, *J* = 8.1 Hz, 2 H), 13.38 (br s, 1 H, NH).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.9, 168.7, 154.6, 150.4, 146.1, 143.4, 140.4, 138.8, 129.4, 129.3, 128.0, 125.1, 120.8, 120.4, 120.3, 119.1, 117.6, 115.8, 114.0, 108.2, 65.5, 60.7.

LR–MS (CI): *m/z* = 542 [MH^+].

HR–MS (EI): *m/z* calcd for $C_{30}H_{27}N_3O_7$: 541.1849; found: 541.1838.

2-Ethoxycarbonylmethoxy-*N*,*N*-bis(ethoxycarbonylmethyl)benzene-1,4-diamine (12b)

To a solution of **3** (6.85 g, 18.6 mmol) in AcOH (60 mL) was added fuming HNO_3 (1.40 g, 1.00 mL, 22.3 mmol) dissolved in AcOH (10 mL) in a dropwise manner at 0 °C over 5–10 min. The reaction was monitored by the disappearance of the starting material on a silica TLC plate. After completion, the reaction mixture was poured on to ice-water. After extraction with CH_2Cl_2, the organic layer was dried over $MgSO_4$ and filtered. After removing of the solvent under reduced pressure, the residue was purified by column chromatography on silica gel (eluent: CH_2Cl_2). Compound **13b** (3.94 g, 51%) was obtained as yellow crystals; mp 76–78 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.25–1.30 (m, 9 H, OCH_3), 4.21–4.27 (m, 10 H, OCH_2, NCH_2), 4.66 (s, 2 H, OCH_2), 6.71 (d, *J* = 8.8 Hz, 1 H, C_6H_3), 7.61 (d, *J* = 2.6 Hz, 1 H, C_6H_3), 7.83 (dd, *J* = 8.8 Hz, *J* = 2.6 Hz, 1 H, C_6H_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.9, 168.0, 147.8, 145.8, 141.1, 119.4, 116.8, 109.1, 66.2, 62.0, 61.9, 61.6, 60.9, 54.5, 14.5, 14.4.

LR–MS (CI): *m/z* = 413 [MH^+].

HR–MS (EI): *m/z* calcd for $C_{18}H_{24}N_2O_9$: 412.1482; found: 412.1493.

To a solution of the nitrated APTRA derivative **13b** (1.65 g, 4.00 mmol) in Et_2O (100 mL) was added Pd/C (5%, 200 mg) and the suspension was left under H_2 at atmospheric pressure. After stirring overnight the mixture was filtered through a layer Celite. The residue, after evaporation of the solvent, was purified by flash column chromatography on silica gel (eluent: EtOAc), giving **12b** (968 mg, 63%) as an oil that turned brown after long-time exposure to air.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.21–1.29 (m, 9 H, OCH_3), 3.49 (br s, 2 H, NH_2), 4.10–4.17 (m, 8 H, NCH_2, OCH_2), 4.25 (q, 2 H, OCH_2), 4.67 (s, 2 H, OCH_2), 6.22 (d, *J* = 2.6 Hz, 1 H, C_6H_3), 6.28 (dd, *J* = 8.4, 2.6 Hz, 1 H, C_6H_3), 6.88 (d, *J* = 8.4, 2.6 Hz, 1 H, C_6H_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.3, 169.2, 151.6, 142.6, 131.4, 122.8, 109.1, 103.4, 66.4, 61.4, 60.4, 54.1, 14.2, 14.1.

LR–MS (CI): *m/z* = 383 [MH^+].

Compound **12a** was prepared in the same way as **12b** giving **13a** (6.20 g, 84%) after nitration (pure on TLC, no additional purifica-

tion) and **12a** (5.71 g, 96%) after reduction (no purification by chromatography necessary).

4-Nitro-2-methoxycarbonylmethoxy-*N*,*N*-bis(methoxycarbonylmethyl)aniline (13a)
Mp 78–80 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.77 (s, 6 H, OCH_3), 3.81 (s, 3 H, OCH_3), 4.22 (s, 4 H, NCH_2), 4.68 (s, 2 H, OCH_2), 6.72 (d, *J* = 8.8 Hz, 1 H, C_6H_3), 7.62 (d, *J* = 2.2 Hz, 1 H, C_6H_3), 7.86 (dd, *J* = 8.8, 2.2 Hz, 1 H, C_6H_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.8, 168.0, 147.4, 145.3, 140.8, 119.1, 116.4, 108.7, 65.7, 53.9, 52.2, 51.9.

LR–MS (CI): *m*/*z* = 371 [MH^+].

HR–MS (EI): *m*/*z* calcd for $C_{15}H_{18}N_2O_9$: 370.1012; found: 370.1012.

2-Methoxycarbonylmethoxy-*N*,*N*-bis(methoxycarbonylmethyl)benzene-1,4-diamine (12a)
Mp 85–88 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.52 (br s, 2 H, NH_2), 3.68 (s, 6 H, OCH_3), 3.72 (s, 3 H, OCH_3), 4.10 (s, 4 H, NCH_2), 4.67 (s, 2 H, OCH_2), 6.20 (d, *J* = 8.8 Hz, 1 H, C_6H_3), 6.28 (dd, *J* = 8.8, 2.2 Hz, 1 H, C_6H_3), 6.87 (d, *J* = 2.9 Hz, 1 H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.2, 170.0, 158.9, 143.3, 131.6, 123.1 (CH), 109.4 (CH), 103.5 (CH), 66.6, 54.3, 52.5, 52.0.

LR–MS (CI): *m*/*z* = 341 [MH^+].

HR–MS (EI): *m*/*z* calcd for $C_{15}H_{20}N_2O_7$: 340.1271; found: 340.1271.

4-Isothiocyanato-2-ethoxycarbonylmethoxy-*N*,*N*-bis(ethoxycarbonylmethyl)aniline (16)
To a solution of **12b** (444 mg, 1.16 mmol), thiophosgene (159 mg, 106 μL, 1.39 mmol) in CH_2Cl_2 (20 mL) at 0 °C was added Et_3N (4 × 100 μL, 288 mg, 2.80 mmol). After stirring for 1.5 h at r.t., H_2O (10 mL) was added. The organic layer was washed with brine and water and dried over $MgSO_4$. After evaporation of the solvent under reduced pressure, **16** (410 mg, 83%) was obtained as a brown oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.22–1.30 (m, 9 H, CH_3), 4.17–4.21 (m, 8 H, OCH_2, NCH_2), 4.28 (q, *J* = 7.1 Hz, 2 H, OCH_2), 4.62 (s, 2 H, OCH_2), 6.64–6.65 (m, 1 H, C_6H_3), 6.81–6.82 (m, 2 H, C_6H_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 207.0, 170.9, 168.2, 149.6, 139.1, 134.2, 124.5, 120.0, 119.9, 111.8, 66.0, 61.4, 60.9, 53.7, 30.94, 14.2, 14.1.

LR–MS (CI): *m*/*z* = 425 [MH^+].

HR–MS (EI): *m*/*z* calcd for $C_{19}H_{24}N_2O_7S$: 424.1304; found: 424.1291.

7-Methoxycarbonylmethoxy-N^6,N^6-bis(methoxycarbonylmethyl)benzothiazolo-2,6-diamine (19a)
A solution of **12a** (1.46 g, 4.30 mmol) and dithiocyanogen (6.00 mmol) in MeOH–Et_2O [prepared from $Pb(SCN)_2$ (1.95 g, 6.00 mmol) and bromine (770 mg, 250 μL, 4.80 mmol) in Et_2O (50 mL)] was stirred for 30 min at r.t. The solvent was evaporated under reduced pressure. The residue was dissolved in EtOAc, heated for a few minutes at reflux temperature and then cooled. The precipitate was filtered affording **19a** (930 mg, 55%) as a grey powder; mp 145 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.60 (s, 6 H, OCH_3), 3.70 (s, 3 H, OCH_3), 4.14 (s, 4 H, NCH_2), 4.83 (s, 2 H, OCH_2), 6.91 (s, 1 H, C_6H_2), 7.44 (s, 1 H, C_6H_2), 9.35 (br s, 2 H, NH_2).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.0, 170.2, 167.0, 149.8, 149.1, 134.7, 124.4, 113.2, 105.3, 66.4, 54.1, 52.6, 52.1.

LR–MS (CI): *m*/*z* = 398 [MH^+].

HR-MS (EI): *m*/*z* calcd for $C_{16}H_{19}N_3O_7S$: 397.0944; found: 397.0946.

7-Ethoxycarbonylmethoxy-N^6,N^6-bis(ethoxycarbonylmethyl)-2,6-diaminobenzothiazole (19b)
To a solution of **3b** (855 mg, 2.24 mmol) and KSCN (325 mg, 3.36 mmol) in MeOH (20 mL) was added bromine (223 mg, 72 μL, 0.63 mmol) in MeOH (5.0 mL) in a dropwise manner at 0 °C. The mixture was stirred for 30 min at r.t and then the solvent was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (20 mL) and then washed with a 10% aq HCl solution, a concentrated $NaHCO_3$ solution, brine and distilled water. The organic layer was dried over $MgSO_4$, filtered and the solvent was partially evaporated. The residue was purified by column chromatography on silica gel (eluent: EtOAc). Compound **19b** was obtained (340 mg, 35%) as a black oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.21–1.27 (m, 9 H, OCH_3), 4.12–4.35 (m, 10 H, OCH_2, NCH_2), 4.70 (s, 2 H, OCH_2), 5.38 (br s, 2 H, NH_2), 6.99–7.03 (m, 1 H, C_6H_2), 7.23 (s, 1 H, C_6H_2).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.6, 171.5, 169.2, 166.4, 150.5, 148.4, 135.9, 125.1, 113.6, 105.8, 66.7, 61.6, 61.0, 60.8, 54.5, 21.4, 14.5.

LR–MS (CI): *m*/*z* = 440 [MH^+].

HR–MS (EI): *m*/*z* calcd for $C_{19}H_{25}N_3O_7S$: 439.1413; found: 439.1402

2-Chloro-7-methoxycarbonylmethoxy-N^6,N^6-bis(methoxycarbonylmethyl)-6-aminobenzothiazole (21)
To a solution of **19a** (397 mg, 1.00 mmol) and $CuCl_2$ (203 mg, 1.50 mmol) in MeCN (20 mL) was added a solution of *tert*-butyl nitrite (90%, 172 mg, 200 μL, 1.50 mmol) in MeCN (10 mL) in a dropwise manner at 0 °C. The mixture was stirred for 1 h at r.t. and then 1 h at 65 °C. The mixture was taken up in EtOAc and washed with an aq HCl solution (6 M) and then with a concd $NaHCO_3$ solution, brine and water. The organic layer was dried over $MgSO_4$, filtered and concentrated. The residue was purified by column chromatography on silica gel (eluent: CH_2Cl_2–EtOAc, 9:1). Compound **21** (113 mg, 28%) was obtained as a grey powder; mp 114–115 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.71 (s, 6 H, OCH_3), 3.79 (s, 3 H, OCH_3), 4.27 (s, 4 H, NCH_2), 4.72 (s, 2 H, OCH_2), 7.27 (s, 1 H, C_6H_2), 7.31 (s, 1 H, C_6H_2).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.8, 178.6, 169.0, 150.5, 146.9, 139.4, 130.2, 111.6, 107.4, 66.2, 54.1, 52.68, 52.3.

LR–MS (CI): *m*/*z* = 417–419 [MH^+].

HR–MS (EI): *m*/*z* calcd for $C_{16}H_{17}ClN_2O_7S$: 416.0445; found: 416.0443.

2-Bromo-7-methoxycarbonylmethoxy-N^6,N^6-bis(methoxycarbonylmethyl)-6-aminobenzothiazole (22)
Compound **22** was prepared by the same procedure as for **21** except that Cu(I)Br was used instead of $Cu(II)Cl_2$. Compound **22** (304 mg, 66%) was obtained as a grey powder; mp 104–105 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.72 (s, 6 H, OCH_3), 3.79 (s, 3 H, OCH_3), 4.26 (s, 4 H, NCH_2), 4.79 (s, 2 H, OCH_2), 7.28 (s, 1 H, C_6H_2), 7.32 (s, 1 H, C_6H_2).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.7, 169.0, 150.3, 148.2, 139.4, 137.1, 131.5, 111.2, 107.1, 66.2, 54.1, 52.4, 52.3.

LR–MS (CI): *m*/*z* = 461–463 [MH^+].

HR–MS (EI): *m*/*z* calcd for $C_{16}H_{17}BrN_2O_7S$: 459.9940; found: 459.9927.

2-Methoxycarbonylmethoxy-*N*⁴-acetyl-*N*,*N*-bis(methoxycarbonylmethyl)benzene-1,4-diamine (24)

To a solution of **3a** (2.20 g, 6.47 mmol) and Et_3N (712 mg, 0.99 mL, 7.11 mmol) in CH_2Cl_2 (50 mL) was added acetyl chloride (420 mg, 462 μL, 6.47 mmol) in CH_2Cl_2 (15 mL) in a dropwise manner at 0 °C. The mixture was stirred for 1 h at r.t. The solvent was evaporated and the residue was used without purification in the next step.

2-Chloro-3-formyl-8-methoxycarbonylmethoxy-7-bis(methoxycarbonylmethyl)aminoquinoline (26) and 2-Chloro-3,6-diformyl-8-methoxycarbonylmethoxy-7-bis(methoxycarbonylmethyl)aminoquinoline (27)

To a mixture of $POCl_3$ (13.8 g, 8.40 mL, 16 equiv) and the residue of the previous step (ca 6.5 mmol) was added DMF (3.76 g, 4.00 mL, 8 equiv) in a dropwise manner at 0 °C. After heating the reaction mixture at 80 °C for 3 h, the mixture was poured in crushed ice and stirred for 3 h. The precipitate was filtered and the strongly colored filtrate was extracted with EtOAc. The combined organic layers were dried over $MgSO_4$, filtered and the solvent was evaporated. The residue from the filtrate was purified by column chromatography on silica gel (eluent: EtOAc). The combined yield of the unseparated compounds **26** (15%) and **27** (21%) was 1.03 g (36%) as a grey-yellow powder.

Compound 26

^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.74 (s, 6 H, OCH_3), 3.78 (s, 3 H, OCH_3), 4.28 (s, 4 H, NCH_2), 4.77 (s, 2 H, OCH_2), 7.08 (s, 1 H, C_6H_2), 7.13 (s, 1 H, C_6H_2), 8.43 (s, 1 H, C_5NH), 10.39 (s, 1 H, CHO).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 189.0, 171.4, 168.0, 155.9, 148.8, 147.5, 141.6, 138.2, 125.2, 123.1, 115.6, 108.4, 65.8, 57.0, 52.8, 52.47.

LR–MS (CI): *m*/*z* = 439–441 [MH^+].

HR–MS (EI): *m*/*z* calcd for $C_{19}H_{19}ClN_2O_8$: 438.0829; found: 438.0828.

Compound 27

^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.65 (s, 6 H, OCH_3), 3.81 (s, 3 H, OCH_3), 4.19 (s, 4 H, NCH_2), 4.89 (s, 2 H, OCH_2), 7.37 (s, 1 H, C_6H_2), 9.68 (s, 1 H, C_5NH), 10.39 (s, 1 H, CHO), 10.88 (s, 1 H, CHO).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 194.4, 189.0, 170.9, 167.8, 159.3, 151.1, 149.2, 147.1, 138.7, 130.1, 126.6, 120.5, 113.3, 65.6, 57.1, 53.0, 52.3.

LR–MS (CI): *m*/*z* = 467–469 [MH^+].

HR–MS (EI): *m*/*z* calcd for $C_{20}H_{19}ClN_2O_9$: 466.0780; found: 466.0793.

7-Methoxycarbonylmethoxy-*N*⁶,*N*⁶-bis(methoxycarbonylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]quinolin-6-amine (30)

Compound **12a** (340 mg, 1.00 mmol) and 5-chloro-4-formyl-3-methyl-1-phenylpyrazole (**28**, 220 mg, 1.00 mmol) were charged in a 10 mL glass tube which was then tightly sealed with an aluminum/Teflon crimp and heated at 180 °C by irradiation at 250 W for 2 min in a CEM-Discover mono-mode microwave apparatus. The cooled solid mixture was dissolved in EtOAc. The organic phase was washed with a concd aq $NaHCO_3$ solution, brine and water. The combined organic layers were dried over $MgSO_4$, filtered and the solvent was evaporated. The residue was purified by column chromatography on silica gel (eluent: CH_2Cl_2 to CH_2Cl_2–EtOAc, 90:10). Compound **30** (75 mg, 20%) was obtained as a yellow powder; mp 89–92 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.68 (s, 3 H, pyrazole-CH_3), 3.78 (s, 6 H, OCH_3), 3.83 (s, 3 H, OCH_3), 4.36 (s, 4 H, NCH_2), 4.84 (s, 2 H, OCH_2), 7.25 (s, 1 H, Ar), 7.29 (s, 1 H, Ar), 7.46–7.53 (m, 3 H, C_6H_5), 8.29 (s, 1 H, Ar), 8.42 (d, *J* = 8.8 Hz, 2 H, C_6H_5).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.9, 168.7, 154.6, 150.4, 146.1, 143.4, 140.4, 138.8, 129.4, 129.3, 128.0, 125.1, 120.8, 120.4, 120.3, 119.1, 117.6, 115.8, 114.0, 108.2, 65.5, 60.7.

LR–MS (CI): 506–508 [MH^+].

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