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SYNTHESIS AND BIOLOGY OF DIMERIC, TRIMERIC AND TETRAMERIC ANALOGUES OF DUOCARMYCIN SA

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Dedicated to Professor Kiyoshi Tomioka on the occasion of his 70th birthday

Abstract – Novel dimeric, trimeric and tetrameric analogues of duocarmycin SA have been prepared by the reaction of *seco*-CBI with di-, tri- and tetracarboxylic acid chlorides in the presence of a base in good yields. The highest bioactivity against cancer cells was observed for the compound obtained from the dicarboxylic acid.

INTRODUCTION

The treatment of cancer is one of the major problems in modern medicine. With the increasing life expectation of the population especially in the developed countries, the risk of getting cancer has increased.¹ Though the medical treatment of cancer has greatly progressed in recent years, there are still several types of cancer which are difficult to cure. A severe obstacle is the lack of discrimination between normal and cancer cells by most of the known chemotherapeutics being the cause of the frequently observed severe side effects.² For increasing the specificity novel approaches have been developed. One option is the targeting of mutations of tumors with small molecules as BRAF inhibitors;³ enhancing the immune response by inhibition of inhibitors of T-cells with immune checkpoint blockade (ICB) is another interesting recently developed option.^{4,5} Application of prodrugs in combination with conjugates of monoclonal antibodies and enzymes (ADEPT)⁶ as well as the use of antibody drug conjugates (ADC)⁷ is also a less toxic treatment alternative.

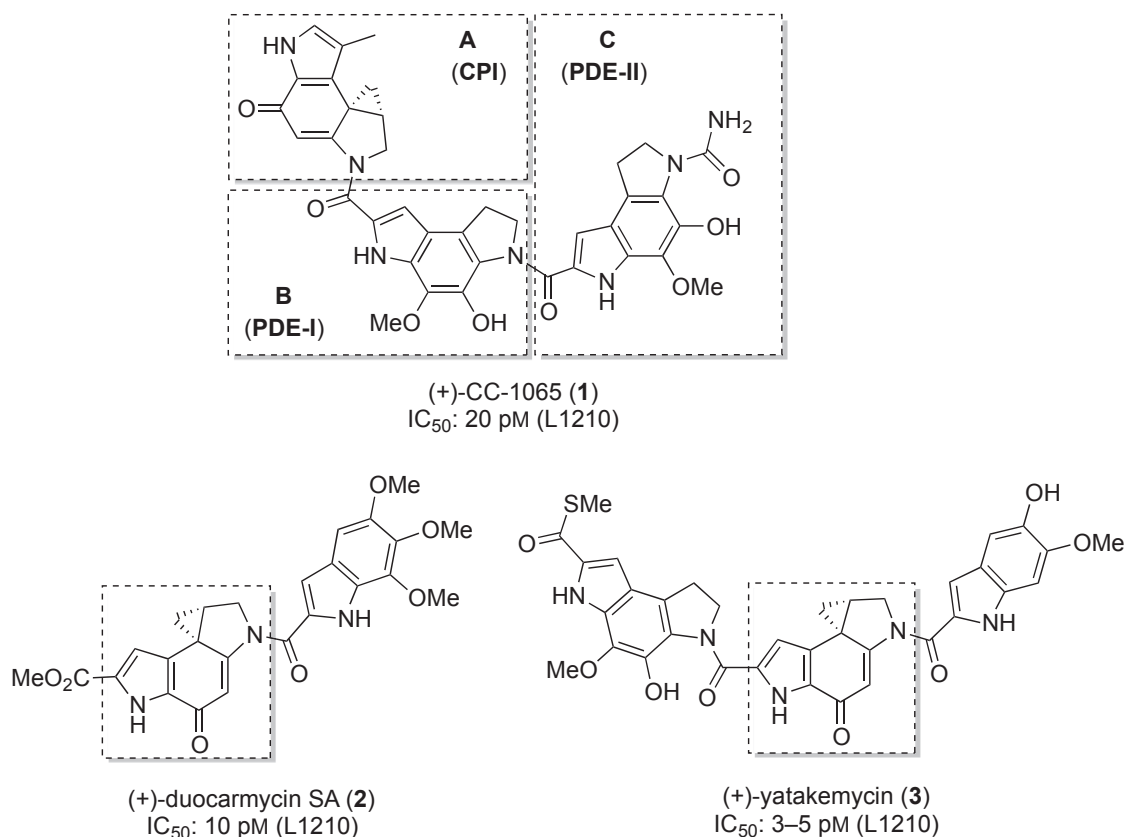


Figure 1. Natural antibiotics (+)-CC 1065 (**1**), (+)-duocarmycin SA (**2**) and (+)-yatakemycin (**3**)

On the other hand, there is always a need for novel potent anticancer agents which might be usable in one of the described ways. At the moment one of our focus lies on the development of drugs which can be used ADCs. The new compounds which we describe in this manuscript are based on the natural antibiotics (+)-CC 1065 (**1**),⁸ (+)-duocarmycin SA (**2**)⁹ and (+)-yatakemycin (**3**)¹⁰ which are very potent cytotoxic agents with IC₅₀-values of 20–3 pM against L1210 cancer cells (Figure 1). They contain a unit with a cyclohexadienone moiety, which acts as a specific alkylating moiety of N3 of an adenine in double stranded DNA. By spectroscopy^{11,12} and other methods it has been shown that these compounds and analogues containing a DNA binding unit first interact very fast with the minor groove of double stranded DNA by a non-covalent incorporation being followed by a slower alkylation of the adenine unit one of the strands. On the other hand the compounds show a low reactivity towards any type of external nucleophiles even the SH-containing tripeptide glutathione.¹³ The most plausible way to explain this fact a proximity effect in the non-covalent DNA drug complex which leads to a reduced barrier of activation.^{14–17} We have recently developed novel dimeric *seco*-analogues of duocarmycin,¹⁸ in which two *seco*-CBI¹⁹ units are connected by a dicarboxylic acid (Figure 2). The cytotoxicity of the dimeric compounds depends on the distance between the two *seco*-CBI-units. Moreover, these compounds can be detoxified by glycosylation.

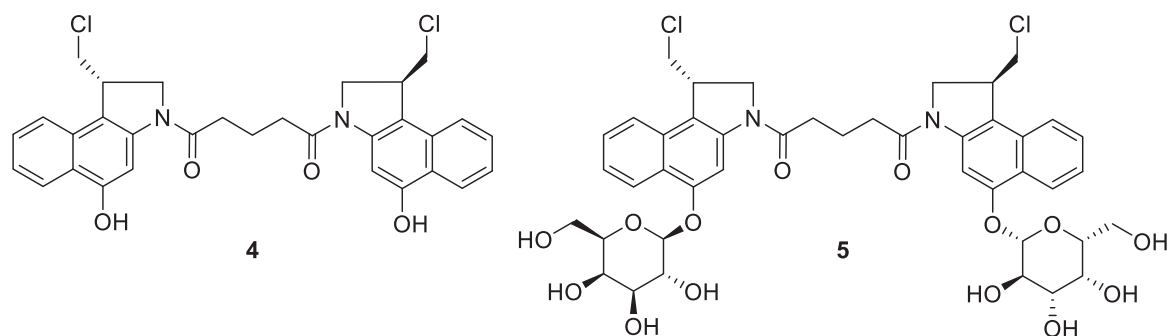


Figure 2. Cytotoxic bis-*seco*-CBI derivative **4** and its much less potent galactosylated counterpart **5**

The most potent compound prepared by us so far is the bis-*seco*-CBI derivative **4** where pentandioate is used as a linker with an IC_{50} -value of 150 fM against A 549 bronchial carcinoma cells. Highly impressive is also the fact that the bis-galactoside **5** is about one million times less cytotoxic than **4**. In our investigations we always employ the *seco*-CBI unit as precursor of the spirocyclopropyl unit since we and other have shown that the *seco*-compounds containing a chloromethyl group are transformed in situ under the conditions of the cell system into the active moiety by a Winstein cyclisation. Compounds of type **4** are rather stable and react only very slowly with external simple nucleophiles. In our investigations using mass spectrometry and CD-spectroscopy we did not observe any interaction with double stranded DNA. We therefore assume that for these compounds another mode of action must be proposed. In cooperation with Sieber et al. we have recently found that the enzyme aldehyde dehydrogenase is a target of the dimeric CBI-compound **4**.²⁰ However, there is a high probability that other enzymes might also be involved. To get a better knowledge about the necessary spatial arrangement of the *seco*-CBI units for high activity we here describe the synthesis of novel compounds containing two, three and four CBI-units connected by di-, tri- and tetra-carboxylic acids via amide bonds. The approach is also in accordance with the perception that including several warheads in one compound might improve its bioactivity. The determination of the bioactivity of the new compounds is performed using a colony-forming ability test.

RESULTS

For our investigations we selected the dicarboxylic acid **6**, the tricarboxylic acids **7** and **8** as well as the tetracarboxylic acid **12**, which are all rather flat. In addition, we also employed the more flexible tricarboxylic acids **9** and **10** and finally the completely flexible tricarboxylic acid **11** (Figure 3).

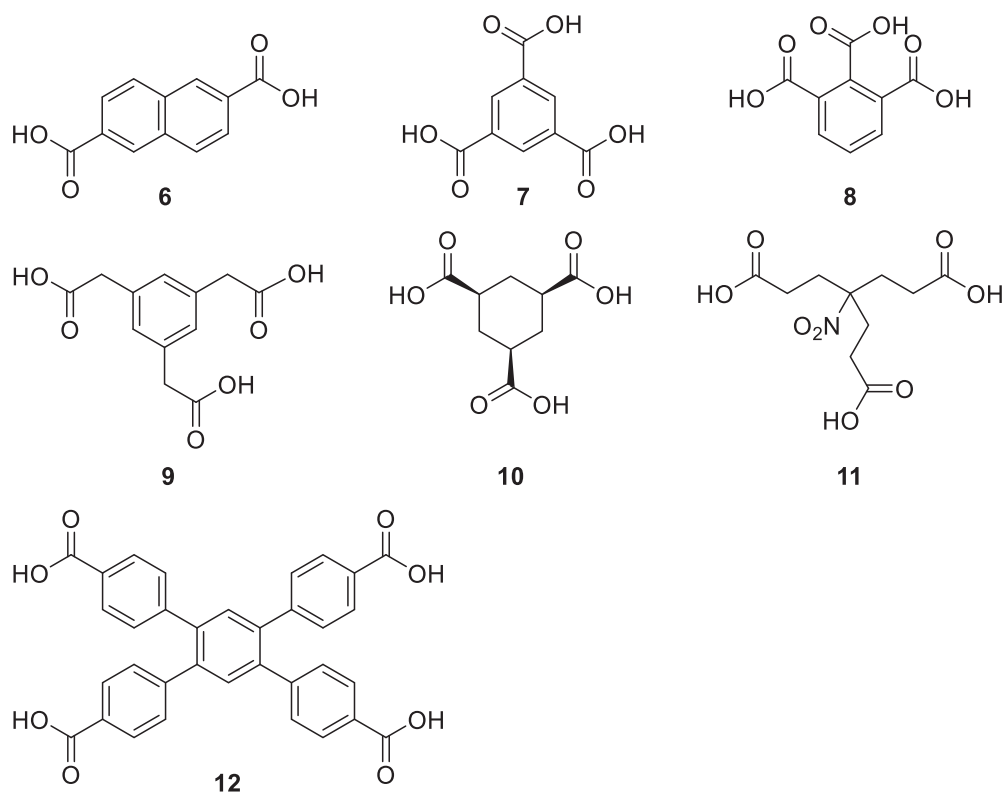
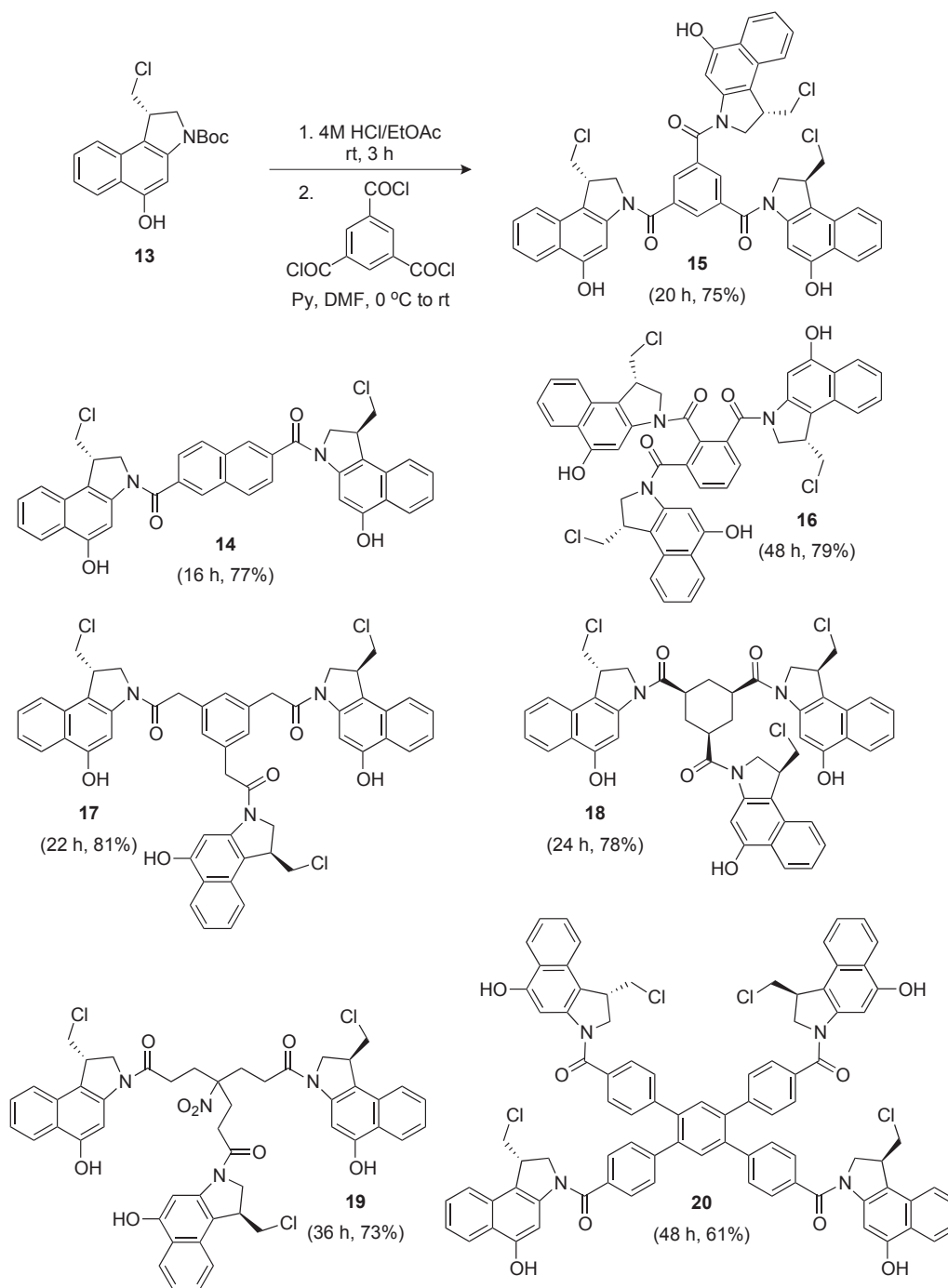


Figure 3. Di-, tri- and tetracarboxylic acid linkers

For the synthesis of the novel dimeric, trimeric and tetrameric CBI derivatives **14** – **20** we used the known *N*-Boc protected enantiopure *seco*-CBI derivative **13**. The necessary removal of the *N*-Boc group in **13** to give the corresponding secondary amine was performed by treatment with 4M HCl in ethyl acetate at rt for 3 h which was then followed without any purification by addition of the acyl chlorides of the different acids **6** – **12** in DMF at 0 °C for 16 – 48 h (Scheme 1).

The shortest reaction time with 16 h was needed for the dichloride of **6** and the longest time for the highly hindered trichloride of **8** and the tetrachloride of **12**. The yields ranged from 61 to 81%. The acid chlorides needed for the transformations were obtained from the corresponding commercially available acids by treatment with oxalyl chloride.

The obtained products were purified by column chromatography and the structure elucidation was performed by mass spectrometry and NMR spectroscopy. We did not observe any unusual steric behavior; however, the NMR spectra measured at room temperature showed the existence of several rotamers. Thus, the NMR data had to be taken at 80 °C to allow a free rotation about the amide bonds.



Scheme 1. Synthesis of the novel dimeric, trimeric and tetrameric CBI derivatives **14** – **20**

In vitro cytotoxicity

The determination of the *in vitro* cytotoxicities of the novel CBI derivatives **14** – **20** was performed on human bronchial carcinoma cells A549 employing a colony-forming ability test that reflects the proliferation capacity of single cells (Table 1). For the assay we exposed the cells to various concentrations of each of the compounds in UC medium (*UltraCulture*TM medium).

Table 1. *In vitro* cytotoxicity^[a] of the *seco*-drugs **14** – **20**

Seco-drug	Number of <i>seco</i> -CBI-units	IC ₅₀ [nM]
14	2	0.37
15	3	120
16	3	220
17	3	4.70
18	3	123
19	3	22.3
20	4	93.0

[a] Determined by HTCFA test

DISCUSSION

The investigation of the cytotoxicity of the *seco*-CBI-derivatives shows that the number of the alkylating moieties in a given compound is not essential for its bioactivity. Thus, compound **14** with only two *seco*-CBI units is the most potent compound within the cohort with an IC₅₀-value of 370 pmol. The other important factor for bioactivity is the steric bulkiness and the flexibility of the compounds. The apparently sterically most demanding compound **16** with three *seco*-CBI-units has indeed the lowest bioactivity with an IC₅₀-value of 220 nmol. This on the other hand is still rather potent in relation to other known cytotoxic compounds, but compared to **4** more than one million times less active. The second lowest bioactivity was found for **18** with an IC₅₀-value of 123 nmol. Astoundingly the huge compound **20** with four *seco*-CBI units is even more cytotoxic with an IC₅₀-value of 93 nmol. Not surprisingly, the rather flexible compounds **17** and **19** are the most active drugs containing three *seco*-CBI-units. All compounds have a higher bioactivity than the simple *seco*-CBI derivative **13** containing a *tert*-butyl group but none of the compounds reaches the cytotoxicity of compound **4** with an IC₅₀-value of 150 fmol. The results clearly confirm that the combination of two *seco*-CBI units with a flexible spacer of defined length is most desirable. This again speaks for a proximity effect.

CONCLUSIONS

Novel dimeric, trimeric and tetrameric *seco*-CBI derivatives **14** – **20** have been prepared, in which a monomeric *seco*-CBI unit **13** is connected to di-, tri- and tetra-carboxylic acids by two, three or four amide bonds. The IC₅₀-values vary from 220 nmol to 370 pmol and are higher than the IC₅₀-value of the most potent compound **4**.

EXPERIMENTAL SECTION

In Vitro Cytotoxicity Assays

Adherent cells of line A549 (human bronchial carcinoma cells) were sown in triplicate in six multiwell plates at concentrations of 10^2 , 10^3 and 10^4 cells per cavity. The culture medium was removed after 24 h and the cells were washed in the incubation medium *UltraCulture*TM (UC, serum-free special medium, Lonza). Incubation with compounds **14** – **20** was then performed in the *UltraCulture*TM medium at 6 – 8 different concentrations for 24 h. All substances were used as freshly prepared solutions in DMSO (Merck, Germany) and diluted with the incubation medium to a final DMSO concentration of 1% in the wells. After 24 h of exposure the test substance was removed and the cells were washed with fresh medium. Cultivation was done at 37 °C and 7.5% CO₂ in air for 9 – 10 days. The medium was removed and the clones were dried, stained with *Löffler's* methylene blue (Merck, Germany) and counted macroscopically. The IC₅₀ values are based on the relative clone forming rate, which was determined according to the following formula: relative clone forming rate [%] = 100 × (number of clones counted after exposure) / (number of clones counted in the control).

Benzene-1,3,5-triyltris{[(S)-1-(chloromethyl)-5-hydroxy-1,2-dihydro-3H-benzo[e]indol-3-yl]methanone} (15): General Procedure. A hydrogen chloride solution (4 M in EtOAc, 5 mL) was added to **13** (70 mg, 0.21 mmol, 1.0 equiv.) at rt. After 3 h the reaction mixture was concentrated under reduced pressure and dried under high vacuum for 1 h. The residue was dissolved in DMF (5 mL) and cooled to 0 °C. Subsequently, pyridine (50 mg, 51 μL, 0.63 mmol, 3.0 equiv.) and the trichloride of **7** (19 mg, 13 μL, 0.073 mmol, 0.35 equiv.) were added with stirring. The resulting mixture was allowed to warm to rt and stirring was continued for 20 h. Upon completion, the reaction was quenched with ice cold water (20 mL) and extracted with EtOAc (4 × 20 mL). The combined organic layers were washed (brine), dried (MgSO₄) and concentrated under reduced pressure. The crude product was purified by flash column chromatography (EtOAc/PE, 1:1) to furnish the target molecule **15** as an orange solid (47 mg, 0.055 mmol, 79% yield). *R_f* = 0.5 (EtOAc/PE, 7:3). *Mp* 135–137 °C. **HPLC** (analytical): Column: Kromosil[®] 100 C18, 250 × 4 mm, 5 μm. Solvent system: MeOH/THF/H₂O = 35:35:30 Flow: 0.8 mL min⁻¹, λ: 254 nm, *t_R*: 23.5 min. **Optical rotation**: $[\alpha]_D^{20}$ -130.0 (*c* 0.73, DMSO). **IR** (ATR): $\tilde{\nu}$ [cm⁻¹] 3155, 3057, 2953, 2921, 2868, 1620, 1579, 1521, 1431, 1392, 1360, 1335, 1262, 1233, 1152, 1118, 1046, 1022, 998, 854, 817, 756, 712, 673, 630; **UV** (MeCN): λ_{\max} (lg ϵ) 332 nm (4.387). **¹H NMR** (400 MHz, DMSO-*d*₆, 85 °C): δ = 10.16 (br s, 3H, OH), 8.16 (d, *J* = 8.5, 3H, 6'-H), 8.04 (s, 3H, 2-H), 7.81 (d, *J* = 8.3 Hz, 3H, 9'-H), 7.69 (br s, 3H, 4'-H), 7.51 (ddd, *J* = 8.2, 6.9, 1.2 Hz, 3H, 8'-H), 7.36 (ddd, *J* = 8.0, 6.9, 1.0 Hz, 3H, 7'-H), 4.47 (dd, *J* = 11.5, 9.1 Hz, 3H, 2'-H_a), 4.17 – 4.07 (m, 6H, 2'-H_b, 1'-H), 3.95 (dd, *J* = 11.0, 3.1 Hz, 3H, 10'-H_a), 3.86 (dd, *J* = 11.1, 7.5 Hz, 3H, 10'-H_b). **¹³C NMR** (101 MHz, DMSO-*d*₆, 85 °C): δ = 3 ×

165.96 (CO), 3 × 153.89 (C-5'), 3 × 141.00 (C-3a'), 3 × 137.39 (C-1), 3 × 129.66 (C-9a'), 3 × 126.86 (C-8'), 3 × 126.72 (C-2), 3 × 122.79 (C-7'), 3 × 122.69 (C-6'), 3 × 122.23 (C-9'), 3 × 122.10 (C-5a'), 3 × 115.10 (C-9b'), 3 × 99.79 (C-4'), 3 × 54.96 (C-2'), 3 × 46.81 (C-10'), 3 × 40.58 (C-1'). **HRMS** (ESI): m/z calcd for C₄₈H₃₆Cl₃N₃O₆ [M+Na]⁺: 878.1550, found: 878.1562.

Naphthalene-2,6-diylbis{[(S)-1-(chloromethyl)-5-hydroxy-1,2-dihydro-3H-benzo[e]indol-3-yl]methanone} (**14**): Prepared according to the general procedure starting from **13** (53 mg, 0.16 mmol, 1.0 equiv.) and acid chloride **6** (20 mg, 0.079 mmol, 0.50 equiv.). The reaction took 16 h to complete and dimeric *seco*-CBI derivative **14** was obtained as a gray solid (40 mg, 0.061 mmol, 77% yield).

R_f = 0.6 (EtOAc). **Mp** 140–142 °C. **HPLC** (analytical): Column: Kromosil® 100 C18, 250 × 4 mm, 5 μm, Solvent system: MeOH/THF/H₂O = 35:35:30, Flow: 0.8 mL min⁻¹, λ: 254 nm, *t_R*: 11.0 min. **Optical rotation**: [α]_D²⁰ -156.0 (*c* 0.66, DMSO). **IR** (ATR): $\tilde{\nu}$ [cm⁻¹] 3056, 2918, 2850, 1624, 1579, 1451, 1416, 1392, 1329, 1250, 1116, 1044, 1021, 984, 850, 823, 761, 752, 714, 673. **UV** (MeCN): λ_{max} (lg ε) 331 nm (4.372). **¹H NMR** (500 MHz, DMSO-*d*₆, 75 °C): δ = 10.18 (br s, 2H, OH), 8.32 (s, 2H, 1-H), 8.19 (d, *J* = 8.4 Hz, 2H), 8.14 (d, *J* = 8.4 Hz, 2H), 7.87 – 7.77 (m, 4H, 3-H, 4-H), 7.52 (ddd, *J* = 8.2, 6.7, 1.3 Hz, 2H, 8'-H), 7.37 (ddd, *J* = 8.1, 6.8, 1.1 Hz, 2H), 4.46 (dd, *J* = 11.5, 9.2 Hz, 2H, 2'-H_a), 4.18 – 4.04 (m, 4H, 2'-H_b, 1'-H), 3.99 (dd, *J* = 11.0, 3.1 Hz, 2H, 10'-H_a), 3.88 (dd, *J* = 11.0, 7.5 Hz, 2H, 10'-H_b). **¹³C NMR** (126 MHz, DMSO-*d*₆, 75 °C): δ = 2 × 167.32 (CO), 2 × 153.90 (C-5'), 2 × 141.23 (C-3a'), 2 × 135.30 (C-2), 2 × 132.63 (C-4a), 2 × 129.79 (C-9a'), 2 × 128.76 (C-4), 2 × 126.98 (C-8'), 2 × 126.44 (C-1), 2 × 124.77 (C-3), 2 × 122.87 (C-7'), 2 × 122.73 (C-6'), 2 × 122.33 (C-9'), 2 × 122.02 (C-5a'), 2 × 115.08 (C-9b'), 2 × 99.73 (C-4'), 2 × 55.02 (C-2'), 2 × 47.00 (C-10'), 2 × 40.44 (C-1'). **HRMS** (ESI): m/z calcd for C₃₈H₂₈Cl₂N₂O₄ [M+H]⁺: 647.1493, found: 647.1499.

Benzene-1,2,3-triyltris{[(S)-1-(chloromethyl)-5-hydroxy-1,2-dihydro-3H-benzo[e]indol-3-yl]methanone} (**16**): Prepared according to the general procedure starting from **13** (60 mg, 0.18 mmol, 1.0 equiv.) and acid chloride **8** (16 mg, 0.060 mmol, 0.34 equiv.). The reaction took 48 h to complete and trimeric *seco*-CBI derivative **16** was obtained as a brown solid (41 mg, 0.048 mmol 79% yield).

R_f = 0.4 (EtOAc/PE, 1:1). **Mp** 127–129 °C. **HPLC** (analytical): Column: Kromosil® 100 C18, 250 × 4 mm, 5 μm, Solvent system: MeOH/THF/H₂O = 30:30:40, Flow: 0.8 mL min⁻¹, λ: 254 nm, *t_R*: 7.0 min. **Optical rotation**: [α]_D²⁰ -226.5 (*c* 0.66, DMSO). **IR** (ATR): $\tilde{\nu}$ [cm⁻¹] 2952, 2869, 1620, 1579, 1522, 1390, 1359, 1335, 1247, 1231, 1153, 1120, 1022, 859, 823, 768, 756, 716, 691, 671. **UV** (MeCN): λ_{max} (lg ε) 329 nm (5.497). **¹H NMR** (500 MHz, DMSO-*d*₆, 75 °C): δ = 10.20 (br s, 1H, OH), 10.06 (br s, 1H, OH), 10.01 (br s, 1H, OH), 8.14 – 8.07 (m, 2H, 2 × 6'-H), 8.05 (d, *J* = 8.4 Hz, 1H, 1 × 6'-H), 7.93 – 7.70 (m, 6H, 9'-H, 4-H, 5-H, 6-H), 7.71 (br s, 2H, 2 × 4'-H), 7.60 (br s, 1H, 1 × 4'-H), 7.56 – 7.44 (m, 3H, 3 × 8'-H), 7.34 (m, 3H, 3 × 7'-H), 4.48 (s, 1H, 1 × 2'-H_a), 4.39 – 4.20 (m, 2H, 2 × 2'-H_a), 4.20 – 3.58 (m,

12H, 3 × 2'-H_b, 3 × 1'-H, 3 × 10'-H_a, 3 × 10'-H_b). ¹³C NMR (126 MHz, DMSO-*d*₆, 75 °C): δ = [165.88, 165.58, 165.40] (CO), [154.11, 154.11, 154.04] (C-5'), 140.60 (C-2), [140.28, 140.28, 140.19] (C-3a'), [135.08, 134.33] (C-1, C-3), 129.85 (C-5), [129.65, 129.65, 129.58] (C-9a'), [127.42, 127.42] (C-4, C-6), [127.05, 127.05, 127.02] (C-8'), [122.86, 122.86, 122.86] (C-7'), [122.86, 122.86, 122.86] (C-6'), [122.40, 122.36, 122.36] (C-9'), [122.17, 122.17, 122.13] (C-5a'), [115.49, 115.21, 115.08] (C-9b'), [99.69, 99.46, 99.46] (C-4'), [54.94, 54.45, 54.14] (C-2'), [46.88, 45.88, 45.88] (C-10'), [41.77, 41.29, 40.51] (C-1'). **HRMS** (ESI): *m/z* calcd for C₄₈H₃₆Cl₃N₃O₆ [M+Na]⁺: 878.1554, found: 878.1562.

2, 2', 2''-Benzene-1,3,5-triyl-tris-(1-[(*S*)-1-(chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benzo[*e*]indol-3-yl]ethan-1-one (17): Prepared according to the general procedure starting from **13** (55 mg, 0.16 mmol, 1.0 equiv.) and acid chloride **9** (18 mg, 0.058 mmol, 0.35 equiv.). The reaction took 22 h to complete and trimeric *seco*-CBI derivative **17** was obtained as a pale brown solid (42 mg, 0.047 mmol, 81% yield).

R_f = 0.4 (CH₂Cl₂/MeOH, 9:1). **Mp** 152–154 °C. **HPLC** (analytical): Column: Kromosil® 100 C18, 250 × 4 mm, 5 μm, Solvent system: MeOH/THF/H₂O = 30:30:40, Flow: 0.8 mL min⁻¹, λ: 254 nm, *t_R*: 73.7 min.

Optical rotation: [α]_D²⁰ -22.5 (*c* 0.66, DMSO). **IR** (ATR): $\tilde{\nu}$ [cm⁻¹] 3058, 2953, 1626, 1579, 1520, 1389, 1361, 1251, 1237, 1153, 1127, 1022, 1000, 857, 754. **UV** (MeCN): λ_{max} (lg ε) 270 nm (5.021), 324 (4.386). **¹H NMR** (300 MHz, DMSO-*d*₆, 75 °C): δ = 10.07 (br s, 3H, OH), 8.10 (d, *J* = 8.3 Hz, 3H, 6'-H), 7.94 (br s, 3H, 4'-H), 7.71 (d, *J* = 8.4 Hz, 3H, 9'-H), 7.47 (ddd, *J* = 8.2, 6.7, 1.3 Hz, 3H, 8'-H), 7.31 (m, 3H, 7'-H), 7.24 (s, 3H, 2''-H), 4.37 – 4.26 (m, 6H, 2'-H), 4.05 (m, 3H, 1'-H), 3.91 (s, 6H, 2-H), 3.90 (dd, *J* = 10.9, 3.2 Hz, 3H, 10'-H_a), 3.70 (dd, *J* = 11.0, 7.9 Hz, 3H, 10'-H_b). ¹³C NMR (125 MHz, DMSO-*d*₆, 75 °C): δ = 3 × 168.11 (CO), 3 × 161.6 (C), 3 × 153.75 (C-5'), 3 × 141.43 (C-3a'), 3 × 134.66 (C-1''), 3 × 129.48 (C-9a'), 3 × 128.01 (C-2''), 3 × 126.58 (C-8'), 3 × 122.60 (C-7'), 3 × 122.15 (C-6'), 3 × 121.92 (C-9'), 3 × 121.49 (C-5a'), 3 × 113.73 (C-9b'), 3 × 99.69 (C-4'), 3 × 52.76 (C-2'), 3 × 46.99 (C-10'), 3 × 42.02 (C-2), 3 × 40.54 (C-1'). **HRMS** (ESI): *m/z* calcd for C₅₁H₄₂Cl₃N₃O₆ [M+Na]⁺: 920.1993, found: 920.2031.

(1*R*, 3*R*, 5*R*)-Cyclohexane-1,3,5-triyltris{[(*S*)-1-(chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benzo[*e*]indol-3-yl]methanone} (18): Prepared according to the general procedure starting from **13** (58 mg, 0.17 mmol, 1.0 equiv.) and acid chloride **10** (17 mg, 11 μL, 0.061 mmol, 0.35 equiv.). The reaction took 24 h to complete and trimeric *seco*-CBI derivative **18** was obtained as a pale brown solid (41 mg, 0.047 mmol, 78% yield).

R_f = 0.5 (EtOAc). **Mp** 144–146 °C. **HPLC** (analytical): Column: Kromosil® 100 C18, 250 × 4 mm, 5 μm, Solvent system: MeOH/THF/H₂O = 35:35:30, Flow: 0.8 mL min⁻¹, λ: 254 nm, *t_R*: 22.6 min. **Optical rotation**: [α]_D²⁰ -12.0 (*c* 0.66, DMSO). **IR** (ATR): $\tilde{\nu}$ [cm⁻¹] 3114, 2953, 1868, 1625, 1579, 1520, 1410, 1395, 1365, 1331, 1259, 1232, 1153, 1125, 1021, 998, 859, 820, 756. **UV** (MeCN): λ_{max} (lg ε) 270 nm

(4.948), 323 (4.282), 355 (3.824). **¹H NMR** (300 MHz, DMSO-*d*₆, 75 °C): δ = 10.11 (br s, 3H), 8.11 (d, *J* = 8.1 Hz, 3H, 6'-H), 8.00 (br s, 3H, 4'-H), 7.79 (d, *J* = 8.2 Hz, 3H, 9'-H), 7.49 (ddd, *J* = 8.3, 6.8, 1.4 Hz, 3H, 8'-H), 7.32 (ddd, *J* = 8.0, 6.8, 1.1 Hz, 3H, 7'-H), 4.51 (m, 3H, 2'-H_a), 4.40 (dd, *J* = 10.9, 2.3 Hz, 3H, 2'-H_b), 4.18 (m, 3H, 1'-H), 4.00 (dd, *J* = 11.0, 3.1 Hz, 3H, 10'-H_a), 3.81 (dd, *J* = 10.9, 8.1 Hz, 3H, 10'-H_b), 3.17 (m, 3H, 2-H), 2.15 (app d, *J* = 12.6 Hz, 3H, 3-H_a), 1.77 (app q, *J* = 12.5 Hz, 3H, 3-H_b). **¹³C NMR** (125 MHz, DMSO-*d*₆, 75 °C): δ = 3 × 172.46 (CO), 3 × 153.99 (C-5'), 3 × 141.7 (C-3a'), 3 × 129.67 (C-9a'), 3 × 126.81 (C-8'), 3 × 122.79 (C-7'), 3 × 122.35 (C-6'), 3 × 122.13 (C-9'), 3 × 121.61 (C-5a'), 3 × 113.95 (C-9b'), 3 × 99.95 (C-4'), 3 × 52.6 (C-2'), 3 × 47.2 (C-10'), 3 × 41.5 (C-1'), 3 × 40.10 (C-2), 3 × 30.4 (C-3). **HRMS** (ESI): *m/z* calcd for C₄₈H₄₂Cl₃N₃O₆ [M+Na]⁺: 884.2036, found: 884.2031.

1,7-Bis((*S*)-1-(chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benzo[*e*]indol-3-yl)-4-(3-((*S*)-1-(chloromethyl)-5-hydroxy-1,2-dihydro-3*H*-benzo[*e*]indol-3-yl)-3-oxopropyl)-4-nitroheptane-1,7-dione (19):

Prepared according to the general procedure starting from **13** (60 mg, 0.18 mmol, 1.0 equiv.) and acid chloride **11** (21 mg, 0.063 mmol, 0.35 equiv.). The reaction took 36 h to complete and trimeric *seco*-CBI derivative **19** was obtained as a pale brown solid (42 mg, 0.046 mmol, 73% yield).

R_f = 0.4 (EtOAc). **Mp** 122–125 °C. **HPLC** (analytical): Column: Kromosil® 100 C18, 250 × 4 mm, 5 μm, Solvent system: MeOH/THF/H₂O = 35:35:30, Flow: 0.8 mL min⁻¹, λ: 254 nm, *t_R*: 17.9 min. **Optical rotation**: [α]_D²⁰ -6.0 (*c* 0.66, DMSO). **IR** (ATR): $\tilde{\nu}$ [cm⁻¹] 3116, 2952, 2914, 1733, 1626, 1579, 1536, 1413, 1392, 1335, 1249, 1153, 1127, 1022, 999, 858, 819, 769, 755, 714, 675. **UV** (MeCN): λ_{max} (lg ε) 267 nm (5.224), 324 (4.504), 341 (4.166), 371 (3.061). **¹H NMR** (300 MHz, DMSO-*d*₆, 75 °C): δ = 10.10 (br s, 3H, OH), 8.11 (d, *J* = 8.4 Hz, 3H, 6'-H), 7.95 (br s, 3H, 4'-H), 7.76 (d, *J* = 8.3 Hz, 3H, 9'-H), 7.48 (ddd, *J* = 8.3, 6.8, 1.2 Hz, 3H, 8'-H), 7.31 (ddd, *J* = 8.1, 6.9, 1.3 Hz, 3H, 7'-H), 4.35 (m, 3H, 2'-H_a), 4.22 (dd, *J* = 10.9, 2.6 Hz, 3H, 2'-H_b), 4.14 (m, 3H, 1'-H), 3.98 (dd, *J* = 10.9, 3.0 Hz, 3H, 10'-H_a), 3.79 (dd, *J* = 10.9, 7.8 Hz, 3H, 10'-H_b), 2.69 – 2.35 (m, 12H, 2-H, 3-H). **¹³C NMR** (125 MHz, DMSO-*d*₆, 75 °C): δ = 3 × 168.74 (CO), 3 × 153.78 (C-5'), 3 × 138.46 (C-3a'), 3 × 129.53 (C-9a'), 3 × 126.59 (C-8'), 3 × 122.61 (C-7'), 3 × 122.10 (C-6'), 3 × 121.92 (C-9'), 3 × 121.45 (C-5a'), 3 × 113.61 (C-9b') 3 × 99.57 (C-4'), 93.09 (C-4), 3 × 52.40 (C-2'), 3 × 47.19 (C-10'), 3 × 40.58 (C-1'), 3 × 29.91 (C-3), 3 × 29.52 (C-2). **HRMS** (ESI): *m/z* calcd for C₄₉H₄₅Cl₃N₄O₈ [M+H]⁺: 923.2378, found: 923.2376.

(1*S*)-1-(Chloromethyl)-3-({4-[2,4,5-tris(4-[(1*S*)-1-(chloromethyl)-5-hydroxy-1*H*,2*H*,3*H*-benzo[*e*]indol-3-yl]carbonyl}phenyl)phenyl]phenyl}carbonyl)-1*H*,2*H*,3*H*-benzo[*e*]indol-5-ol (20): Prepared according to the general procedure starting from **13** (65 mg, 0.19 mmol, 1.0 equiv.) and acid chloride **12** (31 mg, 0.049 mmol, 0.26 equiv.). The reaction took 48 h to complete and tetrameric *seco*-CBI derivative **20** was obtained as a pale brown solid (43 mg, 0.030 mmol, 61% yield).

R_f = 0.4 (EtOAc). **Mp** 164–166 °C, **HPLC** (analytical): Column: Kromosil® 100 C18, 250 × 4 mm, 5 μm, Solvent system: MeOH/THF/H₂O = 35:35:30, Flow: 0.8 mL min⁻¹, λ: 254 nm, t_R : 25.2 min. **Optical rotation**: $[\alpha]_D^{20}$ -5.0 (*c* 0.80, DMSO). **IR** (ATR): $\tilde{\nu}$ [cm⁻¹] 3122, 2950, 2920, 1609, 1582, 1451, 1391, 1360, 1339, 1242, 1153, 1118, 1019, 1005, 846, 756, 718, 706, 674. **UV** (CH₃CN): λ_{max} (lg ε) 341 nm (4.616), 371 (3.916). **¹H NMR** (500 MHz, DMSO-*d*₆, 75 °C): δ = 10.17 (br s, 4H, OH), 8.11 (d, *J* = 8.3 Hz, 4H, 6'-H), 7.76 (d, *J* = 8.5 Hz, 4H, 9'-H), 7.74 (s, 2H, 2''-H), 7.60 (d, *J* = 8.1 Hz, 8H, 2-H), 7.50 (d, *J* = 7.9 Hz, 8H, 3-H), 7.48 (m, 4H, 8'-H), 7.31 (app t, *J* = 7.5 Hz, 4H, 7'-H), 4.35 – 4.23 (m, 4H, 2'-H_a), 4.07 – 3.89 (m, 12H, 2'-H_b, 1'-H, 10'-H_a), 3.76 (dd, *J* = 10.8, 7.7 Hz, 4H, 10'-H_b). **¹³C NMR** (126 MHz, DMSO-*d*₆, 75 °C): δ = 4 × 167.40 (CO), 4 × 153.81 (C-5'), 4 × 141.57 (C-3a'), 4 × 141.22 (C-4), 4 × 138.76 (C-1), 4 × 138.70 (C-1''), 4 × 135.30 (C-1'''), 2 × 132.23 (C-2''), 4 × 129.71 (C-9a'), 4 × 129.57 (C-2), 4 × 126.92 (C-3), 4 × 126.59 (C-8'), 4 × 122.79 (C-7'), 4 × 122.66 (C-6'), 4 × 122.27 (C-9'), 4 × 121.96 (C-5a'), 4 × 114.9 (C-9b'), 4 × 99.8 (C-4'), 4 × 54.96 (C-2'), 4 × 46.76 (C-10'), 4 × 40.51 (C-1'). **HRMS** (ESI): *m/z* calcd for C₈₆H₆₂Cl₄N₄O₈ [M+H]⁺: 1419.3399, found: 1419.3395.

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