Synthesis and evaluation of novel β-lactam inhibitors of human leukocyte elastase

K.P. Koteva, André M. Cantin, W.A. Neugebauer, and E. Escher

Abstract: A series of β-lactam derivatives were synthesized and tested to determine the structure–activity relationship for inhibition of human leukocyte elastase (HLE), a serine protease involved in several degenerative lung and tissue diseases. The most potent IC_{50} values were obtained with neutral hydrophobic 7α-methoxy cephalosporanic acid derivatives. Tryptophanyl-9-fluorenylmethyl ester and *N*-benzhydryl piperazine derivatives of 7α-methoxy cephalosporanic acid represent two novel HLE inhibitors, with length of action persisting beyond 24 h.

Key words: β-lactams, neutrophil elastase, protease inhibitors, peptides.

Résumé : Une série de dérivés de molécules β-lactames fut synthétisée et analysée afin de déterminer la relation structure-activité en tant qu'inhibiteurs de l'élastase neutrophilique humaine (ENH), une protéase impliquée dans plusieurs maladies pulmonaires et tissulaires. Les valeurs inhibitrices (IC_{50}) les plus puissantes furent obtenues avec des dérivés neutres hydrophobes de l'acide 7α-methoxy cephalosporanique. Les dérivés tryptophanyl-9-fluorenylmethyl ester et *N*-benzhydrylpiperazine de l'acide 7α-methoxy cephalosporanique représentent deux nouveaux inhibiteurs de l'ENH, dont l'effet persiste plus de 24 heures.

Mots clés : β-lactames, élastase neutrophilique, inhibiteurs de protéases, peptides.

Introduction

Elastin is a proteinaceous part of the extracellular matrix, commonly called connective tissue. It gives coherence and elasticity to many tissues, such as lung, yellow tendon, and cartilage of joints. Elastin remodeling is controlled by a fine balance between continuous elastin synthesis and breakdown by neutrophil and tissue elastases. Human leukocyte (neutrophil) elastase (HLE, EC 3.4.21.37) secreted by polymorphonuclear neutrophils (PMN) has been considered over two decades as one of the most interesting therapeutic targets in several lung diseases (1). One of the main functions of HLE is the degradation of structural proteins, such as elastin, fibronectin, and collagen (2). Under normal physiological conditions, the lungs are protected from excessive HLE elastolytic activity by endogenous inhibitors such as α1-protease inhibitor (α1-PI) and α2-macroglobulin (α2-M). However, the rate of elastin (and collagen) degradation is greatly enhanced under a variety of clinical conditions. In particular, pulmonary emphysema, rheumatoid arthritis, acute respiratory distress syndrome (ARDS), and cystic fibrosis (CF) (3–5) are characterized by an excessive destruction of connective tissue of the lung and joints. Neutrophil elastase is believed to participate in this process (6). Although α1-PI is present in the CF-lung, it is inactive in most patients, apparently due to oxidative and proteolytic processes (7, 8). Myeloperoxidase present in CF respiratory secretions converts Cl^- to OCl^- in the presence of hydrogen peroxide, inducing a large oxidant burden in the airways (9, 10). Moreover, bronchial secretions from CF-patients can directly inactivate α1-PI through elastase-mediated proteolysis (11). Therefore, individuals with CF have a functional deficiency of airway α1-PI (11, 12). They are especially susceptible to the early development of lung disease. It is reasonable to expect that a selective inhibitor of HLE might be useful for the treatment of emphysema, rheumatoid arthritis, CF, and other diseases. This provides a rationale for developing such inhibitors of low molecular weight for the evaluation of their efficacy to supplement natural inhibitors in diseases involving HLE (13). Several classes of HLE inhibitors have been reported in the past, including *N*-arylbenzylisothiazolinone 1,1-dioxide (14), fatty acids (15), sulfonyl fluorides (16), 4*H*-3,1-benzoxazin-4-one, 4-chloroquinazolines (17), azapeptide *p*-nitrophenyl esters (18), and cephalosporin derivatives. The discovery that neutral cephalosporins are inhibitors of mammalian serine proteinases led Zimmerman and co-workers (19) to evaluate the activity of clavulanic acid and its benzyl esters as inhibitors of HLE. The free acid, known to inhibit the bacterial β-lactamase, was inactive against HLE, but its benzyl ester had good activity against HLE. This observation prompted several investigators to select a large number of β-lactams for evaluation as inhibitors of HLE and other mammalian serine proteases (20–28).

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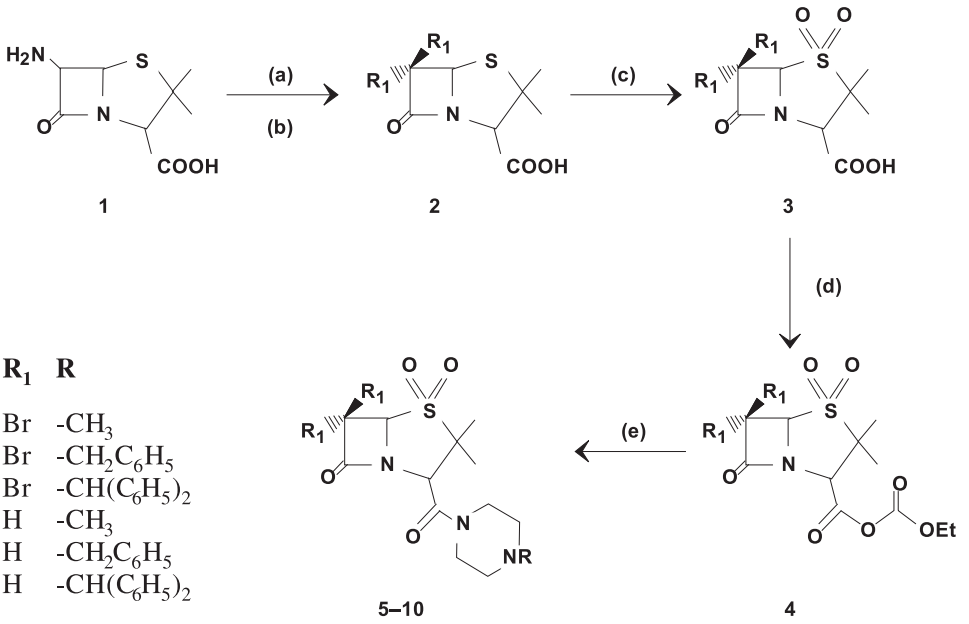
Scheme 1.



	R_1	R
5	Br	$-CH_3$
6	Br	$-CH_2C_6H_5$
7	Br	$-CH(C_6H_5)_2$
8	H	$-CH_3$
9	H	$-CH_2C_6H_5$
10	H	$-CH(C_6H_5)_2$

Reagents: (a) $NaNO_2 \cdot H_2SO_4$; (b) Br_2; (c) $KMnO_4$ / 85% H_3PO_4; (d) EEDQ/DMF; (e) *R*-piperazine

Our objective was to develop a series of novel HLE synthetic inhibitors, including hydrophobic residues covalently linked to β-lactam structures (dibromopenicillanic acid, sulbactam, and 7α-methoxycephalosporin sulfone). Our first approach was to choose three different *N*-substituted piperazines: *N*-methylpiperazine, *N*-benzylpiperazine, and *N*-benzhydrylpiperazine (*N*-Bhpip). They were covalently linked to 6,6-dibromopenicillanic acid-(*S*,*S*)-dioxide (**5**–**7**) and penicillanic acid-(*S*,*S*)-dioxide (sulbactam) (**8**–**10**) (Scheme 1). From this group, *N*-Bhpip was detected as the most potent substituent. Based on the knowledge that *tert*-butyl 7α-methoxycephalosporanate 1,1-dioxide is a potent inhibitor of HNE, we hypothesized that replacing the *tert*-butyl group with hydrophobic amino acids would yield novel potent HNE inhibitors. With a 4-mer combinatorial peptide library, we have shown earlier that hydrophobic amino acids have definite anti-elastase effect (29). The selected amino acids and amino acid esters were covalently linked to 7α-methoxy cephalosporin sulfone (**20**–**31**) and tested for anti-elastase effect.

Experimental

General methods: chemistry

Melting points were measured on a Thomas–Hoover capillary melting point apparatus and were uncorrected. FT-IR spectra were recorded by using PerkinElmer Model 1600 spectrophotometer in chloroform. ^{1}H NMR spectra were recorded on a Bruker 300 spectrophotometer in DMSO-d_6 or $CDCl_3$ referenced to tetramethylsilane as an internal standard at 0.00 ppm. MS spectra were recorded on a high resolution mass spectrophotometer Z AB-IF (electron ionization, 70 eV) and Voyager Biospectrometry Workstation^{TM} based on matrix assisted laser desorption ionization mechanism (MALDI-TOF). Molecular ion peaks for compounds **5**–**10** were obtained. The MS of products **19**–**25**, except product **22**, revealed no molecular ion peak. However, a characteristic homologue $[M]^+$ ion series, subsequently missing – SO_2, – OCH_3, and – $CO\text{-}CH_3COO$, could be assigned. Chromatography generally refers to flash silica gel 60 200–400 mesh (SiliCycle, Quebec, QC, Canada) and TLC plates (Merck Kieselgel 60 F_{254}) with detection using UV or ninhydrine. Thin-layer chromatograms were developed in the following solvent systems: (a) $CHCl_3$:MeOH:AcOH, 95:5:3; (b) BuOH:AcOH:H_2O, 5:2:3; (c) BuOH:pyridine:H_2O, 4:1:1; (d) EtOAc:BuOH:AcOH:H_2O, 2:1:1:1; (e) $CHCl_3$:MeOH:AcOH, 5:3:1; (f) EtOAc:hexane, 8:2; (g) MeOH:$CHCl_3$, 8:2; (h) $CHCl_3$:MeOH, 7:3; or (i) $CHCl_3$:MeOH, 5:5. HPLC wt% analysis was performed on a Waters System 600E Controller at the following conditions: column C18 Bondpack (3.9 × 300 mm), eluent 95:5 (A:B isocratic at 1.0 mL min^{-1}, A = H_2O (0.05% TFA v/v), B = CH_3CN (0.05% TFA v/v)), UV detector at 254 nm. Dimethylformamide (DMF) was distilled over ninhydrine. Dry dichloromethane (DCM) was obtained after filtration over basic alumina. Dicyclohexylcarbodiimide (DCC) was used as 8% solution in DCM. 6,6-Dibromopenicillanic acid-(*S*,*S*)-dioxide was prepared according to refs. 30 and 31. 6-Aminopenicillanic acid (6-APA) and penicillanic acid-(*S*,*S*)-dioxide (sulbactam) were purchased from Sigma-Aldrich (ON, Canada). 7-Aminocephalosporanic acid (7-ACA) was purchased from Fluka (Ronkonkoma, NY). Abbreviations for amino acids follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (32). Other abbreviations are described as follow: *tert*-butyloxycarbonyl (Boc), 9-fluorenylmethyl ester (OFm), benzyl ester (OBz), *tert*-butyl ester (O-*t*-Bu), *o*-nitrophenyl ester (ONp), 9-aminofluorene (NFm), 7α-methoxycephalosporanic acid 1,1-dioxide (CA).

Synthesis of the compounds 5–10 (Scheme 1)

Method A

To a stirred solution of dibromopenicillanic acid-(*S,S*)-dioxide (0.5 g, 1.2 mmol) in DCM (1 mL) was added DCC (1.5 mL, 0.6 mmol) at 0°C. The reaction mixture was then stirred for 20 min. After the filtration of dicyclohexylurea, the solution was used immediately in the next step. *N*-Methylpiperazine (0.55 g, 6.0 mmol) was added and the reaction was carried out at –5 to 0°C for 2 h. Then ice cold water was added and the mixture was stirred for 15 min. The organic layer was separated and the aqueous phase was extracted three times with DCM. The combined DCM extracts were washed with water, citric acid (10%), and brine. After drying over anhydrous sodium sulfate, the solvent was evaporated off under reduced pressure.

Method B

The synthesis was carried out using the method of mixed anhydrides. For this purpose 1-ethoxycarbonyl-2-ethoxy-1, 2-dihydroquinoline (EEDQ) (33) was used as a coupling reagent. *N*-Benzylpiperazine (0.7 g, 4 mmol) was added to a DMF solution of 6,6-dibromopenicillanic acid-(*S,S*)-dioxide (1.5 g, 4 mmol) followed by EEDQ (1.1 g, 4 mmol). The reaction mixture was then stirred for 48 h at room temp. Then ethyl acetate was added and the organic solution was extracted with water, citric acid (10%), and sodium bicarbonate (1 M). After drying over anhydrous sodium sulfate, the ethyl acetate solution was concentrated under vacuum and purified.

Method C

To a DMF solution of 1-methylpiperazine (0.4 g, 4 mmol), *N*-hydroxybenzotriazole (HOBt) (0.65 g, 4 mmol), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3- tetramethyluronium tetrafluoroborate (TBTU) (1.37 g, 4 mmol), and *N*-methylmorpholine (NMM) (8 mmol) were added, followed by sulbactam (1 g, 4 mmol). The reaction mixture was stirred at room temp for 16 h. Then the mixture was diluted with water (70 mL) and extracted three times with ethyl acetate (100 mL). The organic layer was concentrated under reduced pressure and the isolated product purified.

*(2S)-6,6-Dibromo-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0] heptane-2-(4′-methylpiperazine-4′-yl-1′-amido)-4,4-dioxide (**5**)*: Compound **5** was obtained according to method A and purified by preparative thin layer chromatography. Yield: 28%. EI-MS *m/z* ($C_{13}H_{19}Br_2N_3O_4S$, MW 473.19): 473. FT-IR (cm^{-1}): 3400, 1808, 1753, 1672. ^1H NMR (DMSO-d_6) δ: 3.6 (m, 8H) 2.4 (m, 3H), 1.2 (s, 3H), 1.0 (s, 3H).

*(2S)-6,6-Dibromo-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0] heptane-2-(4′-benzylpiperazine-4′-yl-1′-amido)-4,4-dioxide (**6**)*: The synthesis was carried out using method B. Yield: 30%. EI-MS *m/z* ($C_{19}H_{23}Br_2N_3O_4S$, MW 549.28): 550. FT-IR (cm^{-1}): 1802, 1734, 1649, 1633, 1570. ^1H NMR (DMSO-d_6) δ: 7.60 (m, 5H), 4.72 (t, *J* = 6.1 Hz, 2H), 3.61 (m, 8H), 2.43 (s, 3H), 1.60 (s, 3H), 1.39 (s, 3H).

*(2S)-6,6-Dibromo-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0] heptane-2-(4′-benzhydrylpiperazine-4′-yl-1′-amido)-4,4-dioxide (**7**)*: Compound **7** was obtained using method A. Yield: 45%. EI-MS *m/z* ($C_{25}H_{27}Br_2N_3O_4S$, MW 625.38): 625. FT-IR (cm^{-1}): 2982, 2808, 2760, 1817, 1757, 1690, 1662, 1599, 1551. ^1H NMR (DMSO-d_6) δ: 7.35 (m, 10H), 4.20 (t, *J* = 8.2 Hz, 2H), 3.61 (m, 8H), 2.38 (s, 3H), 1.59 (s, 3H), 1.42 (s, 3H).

*(2S)-3,3-Dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-(4′-methylpiperazine-4′-yl-4′-amido)-4,4-dioxide (**8**)*: Compound **8** was obtained according to method C. Yield: 18%. EI-MS *m/z* ($C_{13}H_{21}N_3O_4$ S, MW 315.39): 315. FT-IR (cm^{-1}): 3460, 1790, 1667. ^1H NMR (DMSO-d_6) δ: 3.4–3.8 (m, 8H), 2.4–2.6 (m, 3H), 1.58 (s, 3H), 1.37 (s, 3H).

*(2S)-3,3-Dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-(4′-benzylpiperazine-4′-yl-1′-amido)-4,4-dioxide (**9**)*: The synthesis of compound **9** was carried out using method A. Yield: 20%. EI-MS *m/z* ($C_{19}H_{25}N_3O_4S$, MW 391.49): 391. FT-IR (cm^{-1}): 3465, 1789, 1666. ^1H NMR (DMSO-d_6) δ: 7.2–7.4 (m, 5H), 4.88 (s, 1H), 4.73 (s, 1H), 3.4–3.8 (m, 8H), 2.4–2.6 (m, 3H), 1.61 (s, 3H), 1.38 (s, 3H).

*(2S)-3,3-Dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-(4′-benzhydrylpiperazine-4′-yl-1′-amido)-4,4-dioxide (**10**)*: Compound **10** was prepared using method C. Yield: 40%. EI-MS *m/z* ($C_{25}H_{29}N_3O_4S$, MW 467.59): 467. FT-IR (cm^{-1}): 3479, 1790, 1681, 1651. ^1H NMR (DMSO-d_6) δ: 7.30 (m, 10H), 4.91 (s, 1H), 4.68 (s, 1H), 3.4–3.8 (m, 8H), 2.4–2.6 (m, 3H), 1.62 (s, 3H), 1.42 (s, 3H).

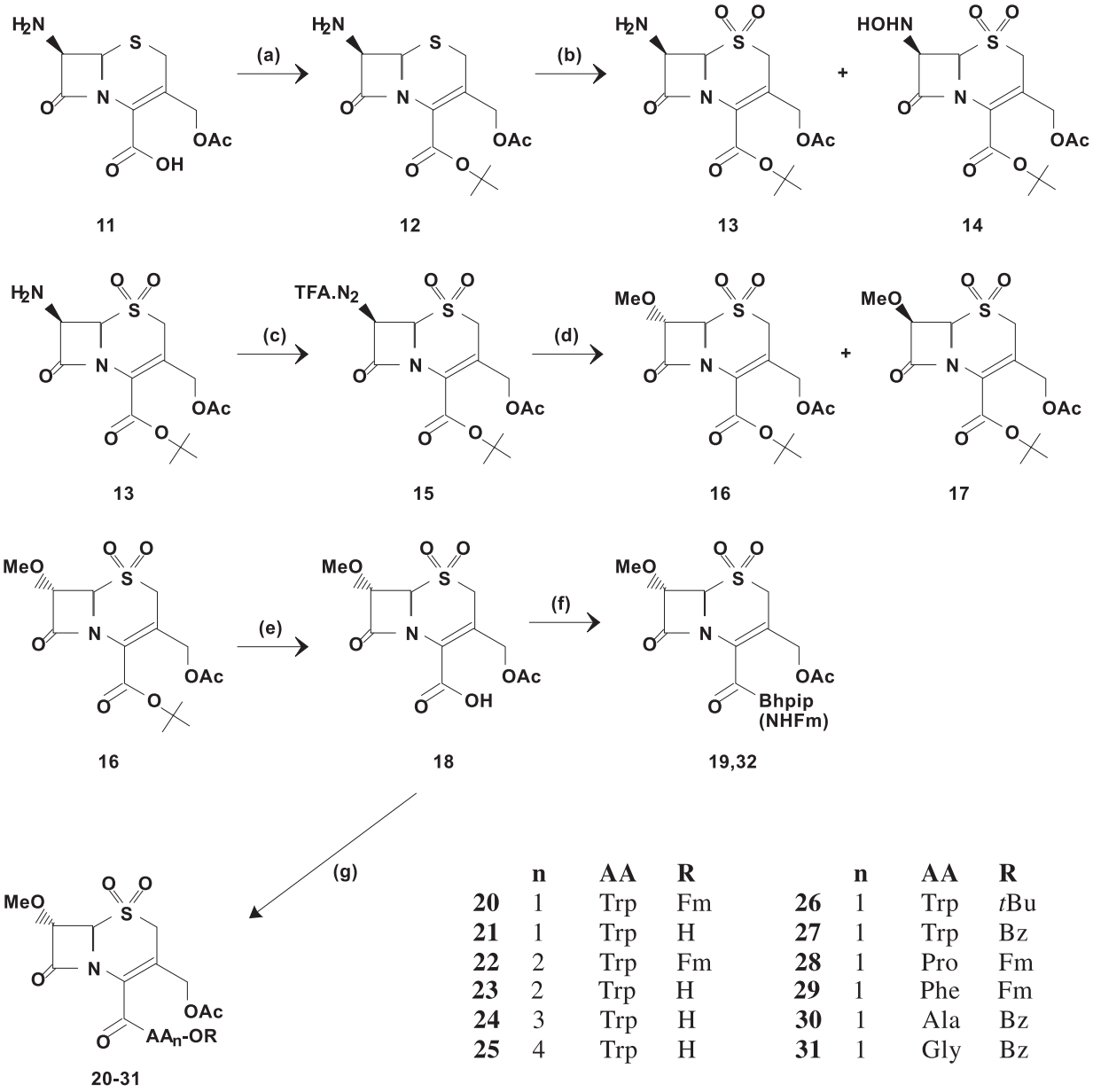
Chemistry of 7-methoxy cephalosporanic acid-1,1-dioxide *tert*-butyl-7β-aminocephalosporanate (12) (Scheme 2)

Dry dioxan was freed from peroxides by passage through a column of neutral alumina. To 100 mL of this solvent was added, by turns, ice cooled sulfuric acid (10 mL), 7-ACA (**11**) (10 g, 0.036 mol), and isobutylene (78.8 mL, 0.73 mol). The mixture was sealed in a pressurized bottle, stirred at room temp for 2 h, and then poured into an excess of ice cooled sodium bicarbonate solution (500 mL). Vigorous release of CO_2 occurred. The pH of the aqueous layer was checked during the quenching to maintain a basic pH (pH 8–9) Extraction with ethyl acetate and evaporation of the solvent gave a brown oil residue, which was then purified through a column of silicagel G 60 A (100 g) with chloroform as the eluent. Yield: 41%, mp 110°C. EI-MS *m/z* ($C_{14}H_{20}N_2O_5S$, MW 328.39): 272 [M – $C(CH_3)_3]^+$, 240 [M – $CO\text{-}CH_3COO]^+$. FT-IR (cm^{-1}): 3459, 2975, 1785, 1723, 1526. ^1H NMR ($CDCl_3$) δ: 5.03 (d, *J* = 13.5 Hz, 1H), 4.94 (d, *J* = 5.2 Hz, 1H), 4.79 (d, *J* = 13.5 Hz, 1H), 4.76 (d, *J* = 5.2 Hz, 1H), 3.55 (d, *J* = 18.3 Hz, 1H), 3.35 (d, *J* = 18.3 Hz, 1H), 2.09 (s, 3H), 1.70 (br s, 2H, NH_2), 1.54 (s, 9H).

tert-*Butyl-7β-aminocephalosporanate-1,1-dioxide (**13**)*

In a 250 mL three-necked round-bottomed flask, equipped with a stirrer and device for external cooling–heating was dissolved **12** (5 g, 0.015 mol) in ethyl acetate (100 mL) at 25°C. The solution was stirred quickly while solid sodium tungstate dihydrate (0.5 g, 0.001 mol) and 30% hydrogen peroxide (6.25 mL, 0.061 mol) were added. The temperature of this biphasic mixture was maintained at 20–25°C with cooling for 2 h. The reaction mixture took on a yellow cast due to the

Scheme 2.



	n	AA	R		n	AA	R
20	1	Trp	Fm	**26**	1	Trp	*t*Bu
21	1	Trp	H	**27**	1	Trp	Bz
22	2	Trp	Fm	**28**	1	Pro	Fm
23	2	Trp	H	**29**	1	Phe	Fm
24	3	Trp	H	**30**	1	Ala	Bz
25	4	Trp	H	**31**	1	Gly	Bz

Reagents: (a) isobutylene/H_2SO_4; (b) H_2O_2/Na_2WO_4; (c) isopropylnitrite/TFA/EtOAc; (d) MeOH/rhodium octanoate dimer / Et_3N; (e) TFA/thioanisol/water; (f) DCC/HOBT/benzhydrylpiperazine(Bhpip)or 9-N_2H-fluorene; (g) H-$(Trp)_n$-OR (R = Fm or H)

formation of the oxidizing species, sodium pertungstate. After 2 h, an additional amount of 30% hydrogen peroxide (1.25 mL, 0.012 mol) was added. After 18 h, an additional hydrogen peroxide amount was added and the batch was left until completion (21 h). When the reaction was deemed complete (TLC), the mixture was diluted with ethyl acetate (150 mL) and cooled to 10°C. The reaction mixture was well stirred while a solution of sodium sulfite (5 g, 0.037 mol) in water (100 mL) was added slowly to decompose any remaining hydrogen peroxide. After 10 min a test for residual peroxide was performed (starch iodide). The biphasic mixture was further cooled to 5°C, and a cold (5°C) solution of aqueous sodium bicarbonate solution (5%) was added to decompose any oxime by-product (**14**). The reaction mixture was stirred for 10 min at 5°C. Stirring was discontinued until the layers were ready to be separated (30 min). The lower aqueous layer was removed and discarded. The upper ethyl acetate product layer was then washed with saturated sodium chloride solution (100 mL). The organic layer was dried over sodium sulfate (5 g), filtered, and cake washed with ethyl acetate (50 mL). The combined filtrate and liquid phases from washing were concentrated under vacuum to give an oily residue, which was further purified through a silicagel G 60A column, using chloroform–methanol (0 → 50% MeOH). Yield: 18.5%. EI-MS

m/z ($C_{14}H_{20}N_2O_7S$, MW 360.51): 273 [M – SO_2 – $C(CH_3)_3$]$^+$, 196 [M – SO_2 – $COOC(CH_3)_3$]$^+$. FT-IR (cm^{-1}): 3360, 2978, 1798, 1731, 1643, 1359, 1154, 1128. ^1H NMR ($CDCl_3$) δ: 5.12 (d, *J* = 14.0 Hz, 1H), 4.91 (d, *J* = 5.2 Hz, 1H), 4.79 (d, *J* = 14.0 Hz, 1H), 4.76 (d, *J* = 4.9 Hz, 1H), 3.66 (d, *J* = 18.6 Hz, 1H), 2.33 (br s, 2H), 2.09 (s, 3H), 1.54 (s, 9H).

tert-*Butyl-7-diazocephalosporanate-1,1-dioxide (**15**)*

A 500 mL three-necked round-bottomed flask equipped with a mechanical agitator and nitrogen bubbler was charged with **13** (0.5 g, 0.0014 mol) in ethyl acetate (15 mL). A freshly prepared solution of isopropyl nitrite (0.16 g, 20 mmol) in DCM (33) was added to the stirred solution at 15–20°C, followed by a catalytic amount of TFA. The batch temperature rose to 31°C over 15 min and the reaction mixture was stirred for 1 h at this temperature. When the diazotization was complete, the batch was diluted with ethyl acetate and concentrated under reduced pressure at 30°C or less to the previous volume (NB: in isolated form the diazo compound may be explosive, however, previous investigators have suggested that this material may be handled safely as a dilute solution in ethyl acetate. Therefore, it was important that the product did not crystallize near the neck of the flask during concentration. The above flash concentration removed most of the excess unreacted isopropyl nitrite, isopropyl alcohol by-product, and methylene chloride.). Afterwards, the batch was diluted with fresh ethyl acetate and used immediately in the next step.

tert-*Butyl-7α-methoxycephalosporanate-1,1-dioxide (**16**)*

Simultaneously to the preparation of the diazocephalosporanate (**15**), rhodium octanoate dimer (20 mg, 0.02 mmol) was dissolved in methanol (1.7 mL) and diluted with ethyl acetate (13 mL). The solution was let to cool. The mixture from the previous step, containing **15**, was cooled to –5°C and vacuum was purged three times with nitrogen. Before the reaction with the compound **15**, triethylamine (0.05 mL) was added to the catalyst mixture, and the solution was stirred for 5 min. The color changed from blue green to purple, probably due to ligand exchange and (or) complexation of the rhodium octanoate dimer with triethylamine. The catalyst solution was added to the solution of diazocompound for about 1 h. The reaction was carried out in an open flask to eliminate nitrogen gas. After 1 h, the batch was concentrated in vacuo, washed with deionized water, containing sodium chloride and phosphoric acid, and then washed with a solution of 10% sodium chloride. The ethyl acetate layer was dried over anhydrous sodium sulfate and then passed through a bed of silicagel G 60A. The silicagel was then washed with ethyl acetate. The batch and wash were combined and concentrated in vacuo to 40 mL. Hexane (8 mL) was slowly added, the crystallization occurred progressively. The crystallization mixture was further concentrated to 5 mL, diluted with hexane (12 mL) over 4 h, let with stirring 4 h at 25°C, and then for 24 h at 0–5°C. The batch was filtered and cake washed with 6 mL of hexane. The residue was dried completely in vacuo. The major product fraction contained 220 mg (24%) of a white crystalline solid, mp 130 to 131°C (EtOAc–hexane), and was identified as *tert*-butyl-7α-methoxycephalosporanate-1,1-dioxide (**16**) on the basis of its spectral characteristics. EI-MS *m/z* ($C_{15}H_{23}NO_8S$, MW 377.42): 273 [M – $COOC(CH_3)_3$]$^+$, 208 [M – SO_2 – $COOC(CH_3)_3$]$^+$, 180 [M – SO_2 – $COOC(CH_3)_3$ – CO]$^+$, 150 [M – SO_2 – $COOC(CH_3)_3$ – CO – OCH_3]$^+$. FT-IR (cm^{-1}): 2978, 1797, 1723, 1693, 1513, 1369, 1157, 1128. ^1H NMR ($CDCl_3$) δ: 5.20 (d, *J* = 14.1 Hz, 1H), 4.99 (d, *J* = 5.0 Hz, 1H), 4.88 (d, *J* = 12.0 Hz, 1H), 4.34 (d, *J* = 13.2 Hz, 1H), 3.99 (d, *J* = 18.8 Hz, 1H), 3.70 (d, *J* = 18.8 Hz, 1H), 3.60 (s, 3H), 2.10 (s, 3H), 1.55 (s, 9H).

*7α-Methoxycephalosporanic acid-1,1-dioxide (**18**)*

A solution of **16** (50 mg, 0.1 mmol) and thioanisol (0.8 mL) in TFA (4 mL) was stirred at 0°C for 30 min and was then evaporated in vacuo. Two additional aliquots of DCM were added and then evaporated to remove most of the residual TFA. The compound was used directly in the next amidation step.

Synthesis in solution of H-Trp-Trp-Trp-Trp-OH (Boc-Trp-OFm) (34), Boc-strategy (Scheme 3)

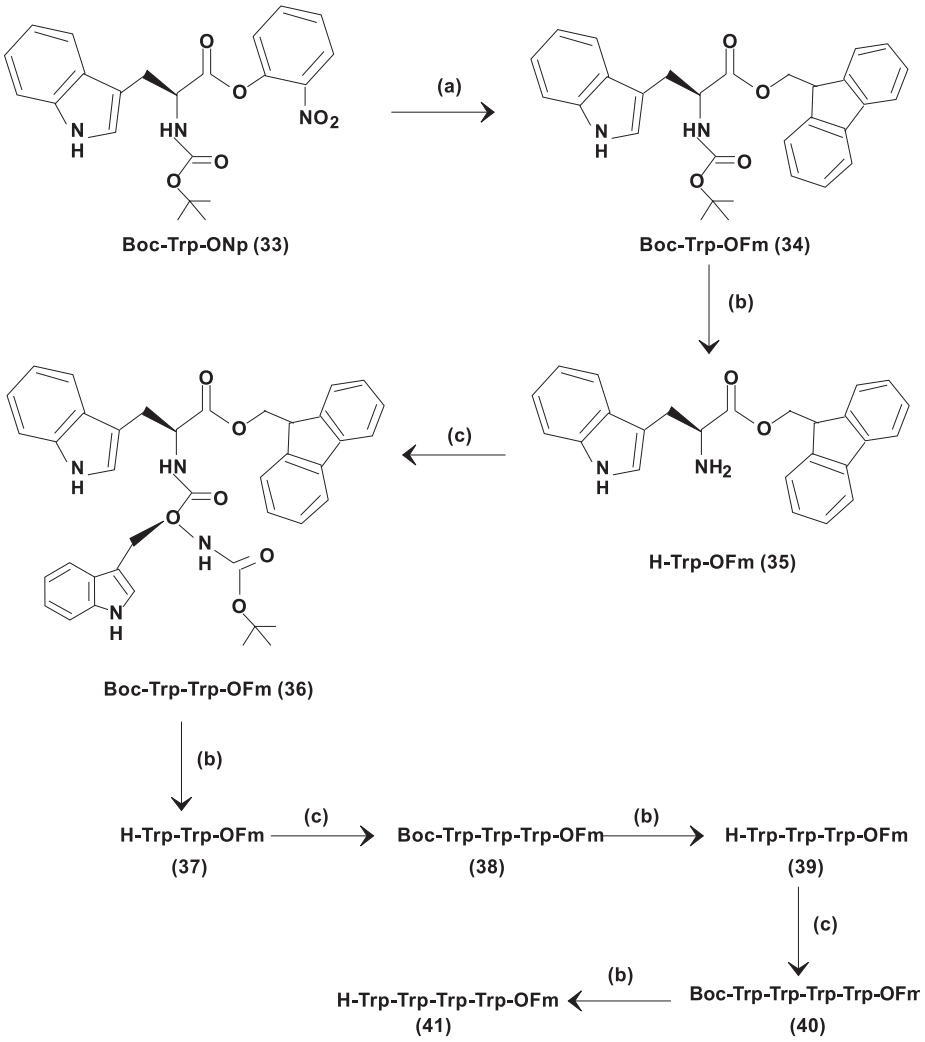
9-Fluorenylmethanol (5.07 g, 0.0258 mol) and imidazol (2.35 g, 0.0345 mol) were added to tetrahydrofurane (40 mL). The suspension was stirred at room temp and *t*-Boc-Trp-ONp (**33**) (10 g, 0.23 mol) was added. Stirring was continued for 18 h. The solvent was then removed under reduced pressure. The residue was dissolved in ethyl acetate, and the solution washed with 2% citric acid solution, sodium bicarbonate solution (0.5 N), and water. The ethyl acetate solution was dried over anhydrous sodium sulfate and evaporated to dryness. The compound was purified by flash chromatography silicagel G 60A, using chloroform as an eluent. Yield 80%. R_f^a: 0.55, R_f^b: 0.84, t_R: 13.6 min. EI-MS *m/z* ($C_{30}H_{30}N_2O_4$, MW 482.58): 482, 178, 130. FT-IR (cm^{-1}): 3409, 3328, 3007, 2979, 2930, 1736, 1699, 1664, 1617, 1590.

*H-Trp-OFm·HCl (**35**)*: Boc-Trp-OFm (**34**) (2.5g, 5 mmol) was dissolved in ether and HCl gas was passed through the solution until precipitation was observed. The amino acid ester hydrochloride was filtered, washed several times with hexane and dried. Yield 95%, mp 200°C. R_f^a: 0.12, R_f^b: 0.80, t_R: 15.4 min. EI-MS *m/z* ($C_{25}H_{22}N_2O_2$, MW 382.47): 382, 178, 152, 130.

*Boc-Trp-Trp-OFm (**36**)*: To a solution of **35** (0.650 g, 1.5 mmol) and HOBt (0.3 g, 1.9 mmol) in DMF (15 mL) was added NMM (0.2g, 1.9 mmol) followed by **33** (0.8 g, 1.9 mmol). The solution was stirred for 4 h at room temp. A 10-fold excess of water was then added, and the obtained suspension was extracted with ethyl acetate. The organic layer and extracts were combined, washed with 0.1% sodium bicarbonate solution, and evaporated under vacuum. The process was followed by TLC and HPLC. The product was further purified by flash chromatography, using silicagel G 60A and $CHCl_3$–MeOH (0 → 50% MeOH) as the eluent. After the purification step, the obtained compound was 1g (91%), mp 88–90°C. R_f^a: 0.60, R_f^b: 0.83, t_R: 20 min. EI-MS *m/z* ($C_{41}H_{40}N_4O_5$, MW 668.80): 669, 569. FT-IR (cm^{-1}): 3409, 3328, 3007, 2977, 2931, 1736, 1697, 1664, 1617, 1591.

*H-Trp-Trp-OFm·HCl (**37**)*: The deprotection was carried out using the procedure already described for **35**. Yield 78%, mp 164 to 165°C. R_f^a: 0.15, R_f^b: 0.78, t_R: 16.5 min. EI-MS *m/z* ($C_{36}H_{32}N_4O_3$, MW 568.68): 569. FT-IR (cm^{-1}): 3409, 3007, 1736, 1697, 1664, 1516.

Scheme 3.



Reagents: (a) 9-fluorenylmethanol/imidazol/THF; (b) ether/HCl(gas); (c)**26**/HOBt/NMM/DMF

*Boc-Trp-Trp-Trp-OFm (**38**)*: The synthesis was carried out using the method described above for **36**. Yield 90%, mp 93–95°C. R_f^a: 0.64, R_f^b: 0.80, t_R: 21.5 min. EI-MS *m*/*z* ($C_{52}H_{50}N_6O_6$, MW 855.01): 855, 475, 374, 146. FT-IR (cm^{-1}): 3404, 3318, 2928, 1736, 1690, 1663, 1509.

*H-Trp-Trp-Trp-OFm·HCl (**39**)*: The Boc-deprotection was carried out according to the method described above. Yield 70%. R_f^a: 0.04, R_f^b: 0.81, t_R: 17.4 min. EI-MS *m*/*z* ($C_{47}H_{42}N_6O_4$, MW 754.90): 755.

*Boc-Trp-Trp-Trp-Trp-OFm (**40**)*: The reaction was carried out according to the method described above. After 16 h water was added and then the extraction with ethyl acetate was done. After purification, the peptide fractions were pooled together and submitted to TLC, HPLC, and EI-MS analysis. The first and major product was Boc-$(Trp)_4$-OFm (M^+ = 1041, MW 1040), and the second one was *tert*-butylated Boc-$(Trp)_4$-OFm (M^+ = 1097, MW 1096). R_f^a: 0.30, R_f^b: 0.70, t_R: 22 min. EI-MS *m*/*z* ($C_{63}H_{60}N_8O_7$, MW 1041.23): 1040, 941, 740, 569, 474, 375.

Solid phase synthesis of H-Trp-Trp-Trp-Trp-OH, Fmoc-strategy

Synthesis was performed using Fmoc-Trp(Boc)-Wang resin, substitution Fmoc-Trp-(Boc)-OH (0.4 mmol g^{-1}, 2.5 eq), DCC (2.5 eq), and HOBt (5 eq), and NMM as a base. The resin cleavage was carried out with TFA–ethanedithiol–water (95:2.5:2.5) for 1 h at room temp. The following compounds were synthesized:

*H-Trp-Trp-Trp-OH (**39**)*: EI-MS *m*/*z* ($C_{33}H_{35}N_6O_4$, MW 576.66): 577.

*H-Trp-Trp-Trp-Trp-OH (**41**)*: EI-MS *m*/*z* ($C_{44}H_{42}N_8O_5$, MW 762.88): 763.

7α-Methoxycephalosporanic acid-1,1-dioxide derivatives (19–31) (Scheme 2)

*CA-Bhpip (**19**)*

To a solution of **18** (42.5 mg, 0.1 mmol) in DMF (3 mL) was added NMM to pH 8, followed by HOBt (19 mg,

0.1 mmol) and DCC (0.5 mL, 0.1 mmol). The mixture was stirred over 30 min at 0°C. *N*-Bhpip (44 mg, 0.17 mmol) was added and the stirring continued overnight. When the reaction was complete DCM was added, followed by water, and the layers were allowed to separate. The organic layer was washed with citric acid solution (2%), water, and brine. After drying over anhydrous sodium sulfate the solvent was removed under reduced pressure. The compound was then purified by column chromatography on silicagel G60 A using chloroform–methanol (0 → 50% MeOH) as a mobile phase. EI-MS *m/z* ($C_{28}H_{31}N_3O_7S$, MW 553.64): 458 [M – SO_2 – CH_3CO]$^+$, 208 [M – SO_2 – CH_3CO – Bhpip]$^+$. FT-IR (cm^{-1}): 3387, 2936, 1791, 1735, 1682, 1522. ^{1}H NMR ($CDCl_3$) δ: 7.60 (m, 10H), 5.20 (d, *J* = 1.6 Hz, 1H), 4.99 (d, *J* = 14.0 Hz, 1H), 4.88 (d, *J* = 1.6 Hz, 1H), 4.34 (d, *J* = 14.0 Hz, 1H), 3.99 (d, *J* = 19.0 Hz, 1H), 3.4–3.8 (m, 8H), 2.10 (s, 3H).

CA-Trp-OFm (20)

To a solution of **35** (47 mg, 0. 1mmol), HOBt (19 mg, 0.1 mmol), and TBTU (40 mg, 0.1mmol) in DMF (2 mL) was added NMM to pH 8 followed by **18** (40 mg, 0.1 mmol). The reaction was checked using TLC. After 7 h the reaction was stopped and 10-fold excess of water was added. The solution was then extracted with ethyl acetate. The ethyl acetate layer was dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure. EI-MS *m/z* ($C_{36}H_{33}N_3O_9S$, MW 683.74): 273 [M – CO – Trp-OFm]$^+$, 208 [M – SO_2 – CO – Trp-OFm]$^+$. FT-IR (cm^{-1}): 1797, 1789, 1769, 1745, 1738, 1731, 1713, 1681, 1667, 1573. ^{1}H NMR (DMSO-d_6) δ: 7.2–7.8 (m, 12H), 6.82 (d, *J* = 2.5 Hz, 1H), 5.18 (d, *J* = 1.6 Hz, 1H), 4.80 (s, 2H), 4.67 (d, *J* = 12.8 Hz, 2H), 4.42 (s, 2H), 4.09 (s, 2H), 3.24 (s, 3H), 2.09 (s, 3H).

CA-Trp-OH (21): The compound was obtained after deprotection of carboxyl function with 15% piperidine in DMF. EI-MS *m/z* ($C_{22}H_{25}N_3O_9S$, MW 507.52): 369 [M – SO_2 – CH_3CO – OCH_3]$^+$, 183 [M – SO_2 – CH_3CO – OCH_3 – CO – Trp]$^+$. FT-IR (cm^{-1}): 2925, 2854, 1789, 1734, 1714, 1667, 1557, 1519.

CA-(Trp)$_2$-OFm (22): The synthesis was carried out according to the method described above. Yield: 31%. EI-MS *m/z* ($C_{47}H_{43}N_5O_{10}S$, MW 869.96): 870. FT-IR (cm^{-1}): 2925, 2854, 1789, 1729, 1714, 1667, 1557, 1519.

CA-Trp-Trp-OH (23): Yield 56%. EI-MS *m/z* ($C_{33}H_{33}N_5O_{10}S$, MW 691.72): 413 [M – SO_2 – Trp – OCH_3]$^+$, 199 [M – SO_2 – OCH_3 – CO – Trp]$^+$. FT-IR (cm^{-1}): 2925, 2854, 1789, 1734, 1714, 1667, 1557, 1519.

CA-(Trp)$_3$-OH (24): 7α-Methoxycephalosporanic acid-1,1-dioxide (**18**) (37 mg, 0.1 mmol) in DMF was added to peptide-resin (250 mg, 0.4 mmol g^{-1}), followed by DCC (0.65 mL, 0.1 mmol), HOBt (17 mg, 0.1 mmol), and a drop of NMM. The synthesis was carried out overnight. After washing and drying, the resin was cleaved with a mixture of TFA–thioanisol–water (3.5:25:0.25) for 1 h at room temp. The peptide was further precipitated in ice cooled ether (200 mL). The solvent was then evaporated and the product lyophilised (purity 98%, HPLC). EI-MS *m/z* ($C_{44}H_{45}N_7O_{11}S$, MW 879.95): 783 [M – SO_2 – OCH_3]$^+$. FT-IR (cm^{-1}): 1790, 1771, 1742, 1670, 1653, 1637, 1593, 1532, 1510.

CA-(Trp)$_4$-OH (25): Yield: 10%. EI-MS *m/z* ($C_{55}H_{55}N_9O_{12}S$, MW 1066.17): 969 [M – SO_2 – OCH_3]$^+$. FT-IR (cm^{-1}): 2926, 2852, 1794, 1774, 1736, 1701, 1666, 1654, 1560, 1523, 1509.

CA-Trp-O-t-Bu (26): Yield: 12%. EI-MS *m/z* ($C_{26}H_{31}N_3O_9S$, MW 561.62): 563, 228 [M – CH_3 – $OCOCH_2$ – Trp-O-*t*-Bu]$^+$, 136 [M – CH_3OCOCH_2 – Trp-O-*t*-Bu – SO_2]$^+$. FT-IR (cm^{-1}): 3370, 1787, 1735, 1679, 1508, 1458, 1385, 1157. ^{1}H NMR (DMSO-d_6) δ: 7.18 (m, 4H), 6.80 (d, *J* = 2.5 Hz, 1H), 5.19 (d, *J* = 1.6 Hz, 1H), 4.81 (t, *J* = 6.4 Hz, 1H), 4.75 (s, 2H), 4.40 (d, *J* = 2.5 Hz, 1H), 4.08 (s, 2H), 3.32 (s, 3H), 2.12 (s, 3H), 1.45 (s, 9H).

CA-Trp-OBz (27): Yield: 10%. EI-MS *m/z* ($C_{29}H_{29}N_3O_9S$, MW 595.63): 596, 484 [M – OCH_3 – SO_2 – CH_3]$^+$, 277 [M – CO – Trp-OBz]$^+$, 137 [M – CO – Trp-OBz – SO_2 – CH_2OCOCH_3]$^+$. FT-IR (cm^{-1}): 3378, 1795, 1741, 1666, 1571, 1135. ^{1}H NMR (DMSO-d_6) δ: 7.20 (m, 5H), 7.18 (m, 4H), 6.80 (d, *J* = 2.8 Hz, 1H), 5.21 (d, *J* = 1.4 Hz, 1H), 4.81 (t, *J* = 7.1 Hz, 1H), 4.75 (s, 2H), 4.42 (d, *J* = 2.5 Hz, 1H), 4.08 (s, 2H), 3.33 (s, 3H), 2.07 (s, 3H).

CA-Pro-OFm (28): Yield: 8%. EI-MS *m/z* ($C_{30}H_{30}N_2O_9S$, MW 594.64): 595, 564 [M – OCH_3]$^+$, 286 [M – Pro-OFm]$^+$. FT-IR (cm^{-1}): 3410, 2960, 1794, 1550. ^{1}H NMR (DMSO-d_6) δ: 7.3–7.8 (m, 8H) 5.21 (d, *J* = 1.4 Hz, 1H), 4.75 (s, 2H), 4.70 (d, *J* = 12.8 Hz, 2H), 4.46 (d, *J* = 15.8 Hz, 1H), 4.21 (t, *J* = 7.0 Hz, 1H), 4.08 (s, 2H), 3.32 (t, *J* = 6.3 Hz, 2H), 3.24 (s, 3H), 2.09 (s, 3H), 1.87 (t, *J* = 7.5 Hz, 2H), 1.60 (m, 2H).

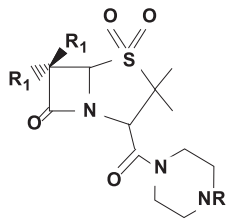
CA-Phe-OFm (29): Yield: 10%. EI-MS *m/z* ($C_{34}H_{32}N_2O_9S$, MW 644.70): 645, 614 [M – OCH_3]$^+$. FT-IR (cm^{-1}): 3401, 1790, 1731, 1679, 1391, 1185. ^{1}H NMR (DMSO-d_6) δ: 7.8–7.1 (m, 13H), 5.22 (d, *J* = 1.4 Hz, 1H), 4.81 (t, *J* = 7.5 Hz, 1H), 4.75 (s, 2H), 4.40 (d, *J* = 12.8 Hz, 1H), 4.13 (s, 2H), 3.3 (s, 3H), 2.11 (s, 3H).

CA-Ala-OBz (30): Yield: 12%. EI-MS *m/z* ($C_{20}H_{22}N_2O_9S$, MW 466.47): 466, 407 [M – $COOCH_3$]$^+$, 343 [M – $COOCH_3$ – SO_2]$^+$, 233 [M – $COOCH_3$ – SO_2 – OBz]$^+$, 135 [M – $COOCH_3$ – SO_2 – CO – Ala-OBz]$^+$. FT-IR (cm^{-1}): 3370, 2983, 1786, 1739, 1662, 1384, 1227, 1156. ^{1}H NMR (DMSO-d_6) δ: 7.4–7.6 (m, 5H), 5.22 (d, *J* = 1.2 Hz, 1H), 4.75 (d, *J* = 12.8 Hz, 2H), 4.43 (d, *J* = 12.0 Hz, 1H), 4.07 (d, *J* = 5.8 Hz, 2H), 4.08 (s, 2H), 3.24 (d, *J* = 18.6 Hz, 3H), 2.10 (s, 3H).

CA-Gly-OBz (31): Yield: 30%. EI-MS *m/z* ($C_{19}H_{20}N_2O_9S$, MW 452.44): 452, 355 [M – $COOCH_3$ – SO_2]$^+$, 261 [M – $COOCH_3$ – SO_2 – OBz]$^+$, 107 (OBz), 135 [M – $COOCH_3$ – SO_2 – CO – Gly-OBz]$^+$. FT-IR (cm^{-1}): 3408, 1786, 1738, 1710, 1665, 1385, 1134. ^{1}H NMR (DMSO-d_6) δ: 7.4–7.6 (m, 5H), 5.22 (d, *J* = 1.2 Hz, 1H), 4.75 (d, *J* = 12.8 Hz, 2H), 4.4 (d, *J* = 12.8 Hz, 1H), 4.13 (d, *J* = 6.9 Hz, 2H), 4.08 (s, 2H), 3.24 (d, *J* = 18.6 Hz, 3H), 2.10 (s, 3H).

CA-NFm (32): Yield: 40%. EI-MS *m/z* ($C_{24}H_{22}N_2O_7S$, MW 482.51): 451 [M – OCH_3]$^+$. FT-IR (cm^{-1}): 3411, 2874, 1788, 1726, 1665, 1368, 1187. ^{1}H NMR (DMSO-d_6) δ: 7.3–7.8 (m, 8H), 5.21 (s, 1H), 4.75 (s, 2H), 4.46 (s, 1H), 4.42 (d, *J* = 12.8 Hz, 1H), 3.35 (s, 3H), 2.01 (s, 3H).

Table 1. Physico-chemical data and anti-elastase activity of penicillin amides **5**–**10**.



	R	R_1	% yield	mp (°C)	t_r (min)	R_f[a]	IC_{50}	Formula
5	CH_3	Br	28	oil	14.7	[b]0.59, [f]0.66	>40	$C_{13}H_{19}Br_2N_3O_4S$
6	$CH_2C_6H_5$	Br	30	oil	10.5	[a]0.09, [f]0.64	inactive	$C_{19}H_{23}Br_2N_3O_4S$
7	$CH(C_6H_5)_2$	Br	45	oil	15.2	[b]0.57, [i]0.66	>40	$C_{25}H_{27}Br_2N_3O_4S$
8	CH_3	H	18	oil	—	[a]0.21, [f]0.48	>40	$C_{13}H_{21}N_3O_4S$
9	$CH_2C_6H_5$	H	20	oil	—	[a]0.15, [f]0.50	inactive	$C_{19}H_{25}N_3O_4S$
10	$CH(C_6H_5)_2$	H	40	oil	—	[a]0.20, [b]0.51	40	$C_{25}H_{29}N_3O_4S$

[a]TLC solvent systems are described in *General methods* section.

Elastase inhibition assay

Neutrophil elastase (HNE) activity was determined with the specific human neutrophil elastase substrate *N*-methoxy-succinyl-alanyl-alanyl-prolyl-valyl-*p*-nitroanilide (MeO-Succ-Ala-Ala-Pro-Val-pNA, Sigma, St. Louis) as described in ref. 12. HNE inhibitory capacity was determined by incubating 20 nM HNE with 0–1 μM inhibitor for 1–24 h at room temp. Residual elastase activity was assayed with MeO-Succ-Ala-Ala-Pro-Val-pNA by measuring the change in absorbance 1 min at 410 nm in a spectrophotometer (model DU7, Beckman Instruments Inc., Montreal, Qc).

Results and discussion

Chemistry

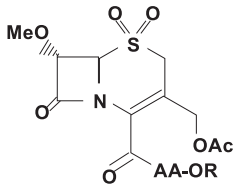
Penicillin derivatives

The commercially available 6-APA, (**1**) (Scheme 1) was first converted to 6,6-dibromopenicillanic acid (**2**) using a diazotization–halogenation procedure (30). A high-yield conversion of **1** to **2** was achieved since the diazo intermediate, once formed, was more easily brominated. The biphase of the diazotization–bromination reaction exploited the solubility of bromine and the 6,6-dibromopenicillanic acid intermediate in organic solvents and the solubility of hydrogen bromide in water. Following Scheme 1, **1** was added to a cooled DCM–sulfuric acid mixture, containing sodium nitrite and bromine, and was converted to the desired 6,6-dibromopenicillanic acid (**2**) in high yield (80%). 6,6-Dibromopenicillanic acid (**2**), once formed, was converted in a very high yield (more than 90%) to the corresponding sulfone (**3**) by a potassium permanganate oxidation (31). The synthesis of the final compounds **5**–**10** was achieved using three different methods: (A) method of symmetrical anhydrides, using dicyclohexylcarbodiimide (DCC) as a coupling reagent, (B) method of mixed anhydrides with the use of EEDQ (33) as a coupling reagent, and (C) amidation via HOBt active ester procedure, using the activating agent TBTU or with DCC. The compounds having formula **5**–**10** obtained after synthesis were 93–99% pure as evidenced by HPLC and TLC analysis (Table 1). They were isolated as micro-crystalline solids or oils. FT-IR, EI-MS, and ^{1}H NMR spectroscopy confirmed the structures.

*Synthesis of 7α-methoxy-cephalosporanic acid-1,1-dioxide (**18**) (Scheme 2)*

The synthesis was carried out according to Scheme 2. 7-ACA (**11**) was used as a starting material. It was apparent that any synthesis of **18** must involve two key chemical transformations, i.e., oxidation of 1-sulfide to the corresponding 1-sulfone and conversion of the 7β-amino group to the 7α-methoxy group. For this reason our preparation followed the procedure described by Blackock et al. (34). Following Scheme 2, the carboxylic function of **11** was protected by esterification with isobutylene, using the method of Stedmann (35). Compound **12** was then oxidized to *tert*-butyl-7-aminocephalosporanate-1,1-dioxide (**13**). Direct oxidation of 7-ACA *tert*-butyl ester to the corresponding sulfone caused some unique problems due to the potential for *N*-oxidation of the 7-amino group. As in step one, we believed that protection from *N*-oxidation might come via protonation of the amine prior to the oxidation step. Our initial attempts to oxidize compound **12** using *m*-chloroperoxybenzoic acid (*m*-CPBA) were unsuccessful. However, using sodium tungstate as a catalyst and 30% hydrogen peroxide solution we managed to obtain the sulfone, but in very low yield (18.5%). Some *N*-oxidation occurred to form 20% of a nearly insoluble and crystalline by-product, 7-hydroxyimino sulfone derivative (**14**) of 7-ACA *tert*-butyl ester. Synthesis of *tert*-butyl-7α-methoxycephalosporanate-1,1-dioxide (**16**) was the next step. For the diazotization–methoxylation procedure we followed the method previously described (34). The use of isopropyl nitrite as the diazotizing reagent facilitated the workup, as the by-product (isopropyl alcohol) reaction could be readily removed from the reaction mixture by vacuum replacement concentration. The fast reaction rate of methoxylation ensured a higher insertion yield, but slowing down the rate with a reduced methanol and catalyst charge increased stereoselectivity. For this reason cold (0°C) ethyl acetate solution, containing rhodium octanoate dimer, methanol, and thriethylamine was added slowly over 1 h to the cold solution of the diazocomponent. *tert*-Butyl-7β-methoxy-cephalosporanate (**17**) was also obtained as a by-product.

Table 2. Physico-chemical data and antielastase activity of cephalosporin amides **20**–**31**.



	AA	R	% yield	mp (°C)	R_f[a]	t_R (min)	IC_{50}	Formula
20	Trp	Fm	24	foam	[b]0.65, [c]0.84	8.7	0.8	$C_{36}H_{33}N_3O_9S$
21	Trp	H	42	oil	[a]0.01, [b]0.66	—	>40	$C_{22}H_{25}N_3O_9S$
22	$(Trp)_2$	Fm	37	oil	[a]0.05, [b]0.60	10.0	15	$C_{47}H_{43}N_5O_{10}S$
23	$(Trp)_2$	H	98	oil	[b]0.72, [c]0.80	13.7	>40	$C_{33}H_{33}N_5O_{10}S$
24	$(Trp)_3$	H	31	oil	[a]0.09, [b]0.67	17.9	>40	$C_{44}H_{45}N_7O_{11}S$
25	$(Trp)_4$	H	56	oil	[b]0.95, [c]0.80	18.1	>40	$C_{35}H_{55}N_9O_{12}S$
26	Trp	*t*-Bu	10	oil	[a]0.10, [b]0.60	15.2	<40	$C_{26}H_{31}N_3O_9S$
27	Trp	Bz	10	oil	[a]0.11, [b]0.58	11.4	10	$C_{29}H_{29}N_3O_9S$
28	Pro	Fm	8	oil	[d]0.76, [e]0.41	12.7	>40	$C_{30}H_{30}N_2O_9S$
29	Phe	Fm	10	oil	[a]0.06, [b]0.62	9.6	<40	$C_{34}H_{32}N_2O_9S$
30	Ala	Bz	12	oil	[b]0.07, [h]0.20	10.9	>40	$C_{20}H_{22}N_2O_9S$
31	Gly	Bz	30	oil	[a]0.05, [h]0.17	10.7	<40	$C_{19}H_{20}N_2O_9S$

[a]TLC solvent systems are described in *General methods* section.

The free acid **18** was obtained by cleavage of *tert*-butyl ester group with TFA–thioanisol. Compound **18**, a light yellow oil, was used without isolation in the next amidation step.

Synthesis in solution of H-Trp-Trp-Trp-Trp-OH, Boc-strategy (Scheme 3)

A method for the synthesis of tetra-tryptophanyl-9-fluorenylmethyl ester was developed. From numerous methods proposed for the preparation of amino acids esters, we selected the imidazol-catalyzed transesterification of a commercially available protected amino acid active ester, Boc-Trp-ONp (33). Following Scheme 3, in the first step the *o*-nitrophenylester group was substituted with the 9-fluorenylmethyl ester group using the method of Bednarek and Bodansky (36). The *tert*-butyloxycarbonyl amino protection of **34**, **36**, **38**, and **40** could be cleaved either by TFA or by gaseous HCl as the fluorenylmethyl ester group remains unaffected under these conditions. For the synthesis we chose gaseous HCl Boc-cleavage. The next tryptophanyl residue was then attached using Boc-Trp-ONp ester. The coupling was carried out using HOBt–active ester in situ formation in the presence of NMM as a base. It is interesting to note that the fluorenylmethyl group contributes to the solubility of the protected peptides in organic solvents. Thus, fluorenylmethyl esters might offer some advantages in peptide synthesis, particularly because they are readily removable under mild conditions (15% piperidine in DMF).

Solid phase synthesis of H-Trp-Trp-Trp-Trp-OH, Fmoc-strategy

Synthesis was performed using Fmoc-Trp(Boc)-Wang resin, substitution Fmoc-Trp(Boc)-OH (0.4 mmol g^{-1}, 2.5 eq), DCC (2.5 eq), HOBt (5 eq), and MMM as a base. The cleavage from the resin was carried out with TFA–ethanedithiol–water (95:2.5:2.5) for 1 h at room temp. The final product (**41**) was purified by HPLC.

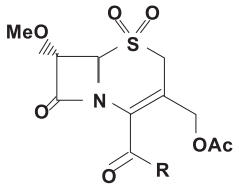
7α-Methoxycephalosporanic acid derivatives (19–32) (Scheme 2)

The synthesis of the compounds with the formula **19**–**32** was achieved in low to moderate yield using the method of active esters. The predicted structures were confirmed by FT-IR, NMR, and EI-MS spectroscopy. Physico-chemical data for the compounds from this series are shown in Tables 2 and 3.

In vitro activity

In vitro inhibition of HLE activity by the target compounds is shown in Tables 1, 2, and 3 as nominal IC_{50} values (μM). From the results in Table 1, it can be seen that the compounds prepared on the basis of the penicillin structures (dibromopenicillanic acid and sulbactam) are very poor time-dependant inhibitors of HLE. From this series of compounds, we chose *N*-Bhpip as the most promising substituent and it was determined that compound **19**, on the basis of cephalosporin structures, had a strong anti-elastase effect (IC_{50} = 0.8 μM) (Table 3). Substitution of *N*-Bhpip with 9-aminofluorene gave compound **32**, which exhibited poor anti-elastase activity. Biological data for the compounds with amino acids and amino acid esters covalently linked to the cephalosporin (structures **20**–**31**) are shown in Table 2. Replacement of the *tert*-butyl ester group in **16** with tryptophanyl-9-fluorenylmethyl ester gave compound **20**, possessing the same anti-elastase activity as **19**. The free carboxylic group on **21** gave a diminished anti-elastase effect. The addition of more tryptophanyl residues gave poor HLE inhibitors (**22**–**25**), as demonstrated by the absence of activity in the compound with up to four tryptophanyl residues. Both the tryptophanyl residue and the Fm ester group were found to be essential for anti-HLE activity. Replacement of the Fm ester group in **20** with *t*-Bu (**26**) and benzyl (Bz) (**27**) ester groups gave poor anti-elastase inhibitors. Furthermore, the importance of tryptophanyl was

Table 3. Physico-chemical data and antielastase activity of cephalosporin sulfones.



	R	% yield	mp (°C)	R_f[a]	t_R (min)	IC_{50}	Formula
16	O-*t*-Bu	24	130 to 131	[a]0.53, [f]0.55	—	1.0	$C_{15}H_{23}NO_8S$
19	Bhpip	42	foam	[a]0.15, [b]0.54	10.9	0.8	$C_{28}H_{31}N_3O_7S$
32	NHFm	30	oil	[f]0.29, [g]0.73	—	<40	$C_{24}H_{22}N_2O_7S$

[a]TLC solvent systems are described in *General methods* section.

demonstrated by the substitution of tryptophanyl 9-fluorenylmethyl ester with prolyl-9-fluorenylmethyl ester and phenylalanyl-9-fluorenylmethyl ester yielding compounds **28** and **29**, respectively, with reduced anti-elastase activity. Finally, the substitution of tryptophanyl-9-fluorenylmethyl ester with commercially available alanyl benzyl ester (**30**) and glycyl benzyl ester (**31**) gave the same results.

Conclusion

We have demonstrated that replacement of *t*-butyl ester group in 7α-methoxycephalosporanic acid 1,1-dioxide with tryptophanyl-9-fluorenylmethyl ester (compound **20**) yields a strong HLE inhibitor with a length of action beyond 24 h. Deprotection of the carboxylic function resulted in loss of activity. Replacement of tryptophan with tetra-tryptophan or other amino acids, as well as replacement of tryptophanyl-9-fluorenylmethyl ester with other tryptophanyl-esters led to compounds with diminished anti-elastase effect. However, replacement of tryptophanyl-9-fluorenylmethyl ester with the hydrophobic *N*-Bhpip gave compound **19** having the same inhibitory effect as **20**, both of which are novel HLE inhibitors.

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