Discovery of a Potent Adenine-Benzyltriazolo-Pleuromutilin Conjugate with Pronounced Antibacterial Activity against MRSA

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Discovery of a potent adenine-benzyltriazolo-pleuromutilin conjugate with pronounced antibacterial activity against MRSA

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Abstract

Conjugation of pleuromutilin is an attractive strategy for development of novel antibiotics and the fight against multi-resistant bacteria as the class is associated with low rates of resistance and cross-resistance development. Herein, the preparation of 35 novel (+)-pleuromutilin conjugates is reported. Their design was based on a synthetically more efficient benzyl-adaption of a potent lead, but still relied on the Cu(I)-catalysed alkyne-azide [3+2] cycloaddition for conjugation onto pleuromutilin. Their antibacterial activity was evaluated against the multi-resistant *Staphylococcus*

aureus strain USA300 for which they displayed moderate to excellent activity. Compound **35**, bearing a para-benzyladenine substituent proved particularly potent against USA300 as well as additional strains of MRSA and displayed as importantly no cytotoxicity in four mammalian cell lines. Structure-activity relationship analysis revealed that the purine 6-amino is essential for high potency, likely due to strong hydrogen bonding with the RNA backbone of C2469 as suggested by a molecular model based on the MM-GBSA approach.

Introduction

Antibiotic resistance poses an imminent threat to global health.[1,2] The evolutionary ability of bacteria to develop resistance towards small molecules coupled with the heavy expenses of bringing a novel drug to market has granted them one of the lowest financial rentabilities of commercially developed drugs. Consequently, many large pharmaceutical companies discontinued their antimicrobial projects around the new millennia. Academic research and development of new antibiotics with low inherent rates of resistance and cross-resistance are thus acute. The antibiotic class of pleuromutilins has proven to possess these resilient properties.[3] The diterpene natural product (+)-pleuromutilin (**1**, Figure 1), which the class is based upon, was first isolated in 1951.[4,5] Since then, a large number of semi-synthetic pleuromutilin conjugates have been synthesised of which four have reached the market (Figure 1).[6] These include two veterinary agents, tiamulin[7] (**2**, Denagard®, 1975) and valnemulin[8] (**3**, Econor®, 1998) as well as the clinical agents retapamulin[9] (**4**, Altargo®, 2007) approved for short-term treatment of skin and soft tissue infections (SSTIs) and finally the very recent lefamulin[10-12] (**5**, Xenleta®, 2019) approved for the treatment of community-acquired pneumonia by oral administration.[10-12]

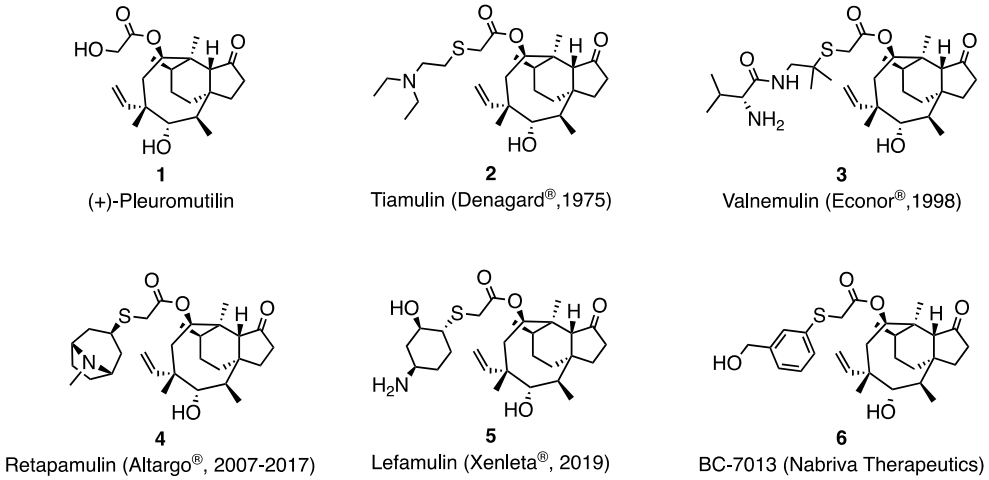


Figure 1. Structures of clinically approved and relevant pleuromutilins conjugates.

The pleuromutilin class is especially potent towards Gram-positive pathogens of the *Staphylococci*, *Mycoplasmas* and *Streptococci* species.[3] The antibacterial effect of pleuromutilins arises from their ability to inhibit bacterial protein synthesis by binding to the A- and P-site of the peptidyl transferase centre (PTC) located in the large ribosomal subunit (50S). The orientation and binding mode of pleuromutilins have been thoroughly elucidated by footprinting studies,[13-15] X-ray crystallography[16-18] and molecular modelling.[16,19] From these, it has been established that the tricyclic mutilin core resides in the A-site of the PTC (Figure 2; red circle), while the C14 substituent (glycolic ester, or modifications thereof, blue ellipse) protrudes into the P-site.[13,16,17,20,21] This hinders tRNA association, positioning and the crucial 3′-end tRNA A- to P-site rotary motion required for peptide bond formation (Figure 2 A).[13,16-19,22,23]

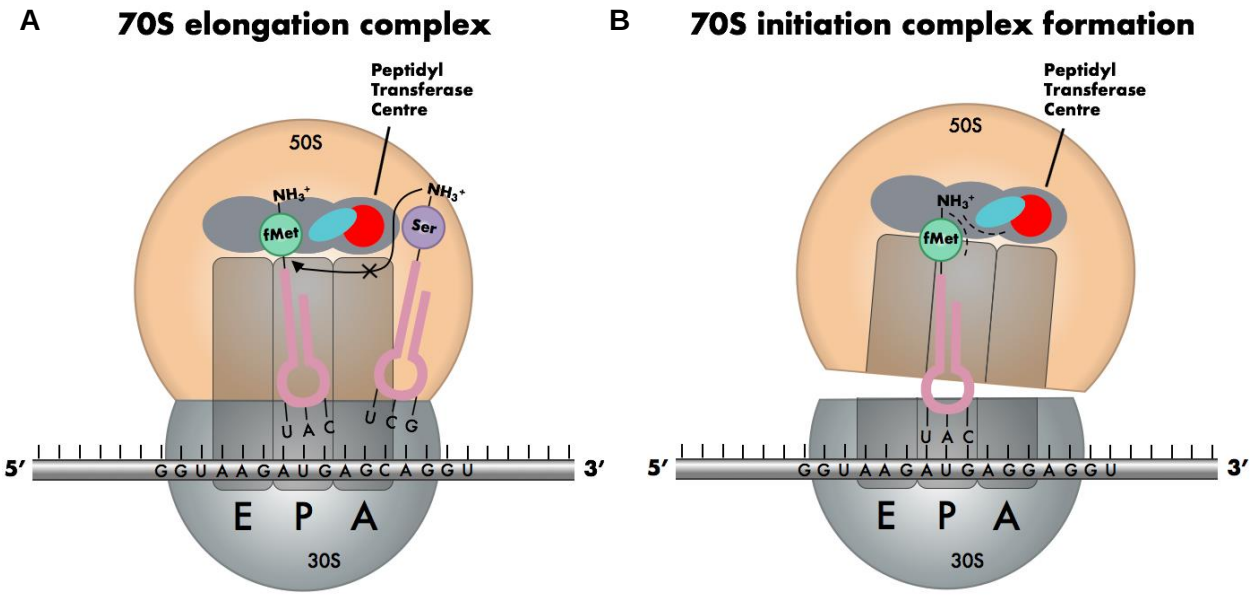


Figure 2. Pleuromutilin mode of binding and actions. Tricyclic mutilin core: red circle; C14 substituent: blue ellipse. (A) Inhibition of tRNA association and peptide bond formation. (B) Inhibition of initiation complex formation by sterical hindrance.

Furthermore, pleuromutilins have also shown to inhibit the formation of the 70S initiation complex (Figure 2 B). This is based on the fact that radiolabelled tiamulin is unable to bind once translational elongation has begun.[24] Secondly, addition of tiamulin (**2**) to intact cells leads to ineffective initiation complexes,[24] and lastly, retapamulin (**4**) has been shown to partially inhibit fMet-tRNA binding which in turn disrupts the formation of the 70S initiation complex.[20] Among protein synthesis inhibitors, the aforementioned ability is unique to pleuromutilins, and may thus help explain its lack of clinically relevant cross-resistance.[20]

In July 2018, retapamulin (**4**) was withdrawn by GlaxoSmithKline,[25] which leaves the market without a pleuromutilin conjugate optimized for topical use. A promising candidate for potentially filling this gap is BC-7013 (**6**), currently in development by Nabriva Therapeutics for the treatment of uncomplicated skin and skin structure infections (uSSSIs).[26-29] It displays strong activity towards Gram-positive bacteria, including methicillin resistant *Staphylococcus* aureus (MRSA), coagulase-negative *Staphylococci* (CoNS) and *Streptococcus Pyogenes* strains with minimal inhibitory concentrations (MIC) of 0.03, 0.06 and 0.03 μg mL^{-1}, respectively. It has successfully passed phase I clinical trials.

In 2014 we identified compound **7**[14] (Figure 3) to possess excellent activity towards the clinically prevalent and virulent strain MRSA USA300[30-32] signified by a MIC of 0.06 μg mL^{-1}.[14] Unfortunately, synthesis of **7** was fairly inefficient and challenging, signified by a poor convergent total yield of 7% largely due to the Chan−Lam−Evans-modified Ullmann condensation used to form the adenine-phenyl bond.[19] To evade this, we decided to exchange the phenyl-scaffold for a benzyl-layout (Figure 3) which allows for easier derivatisation and thereby more efficient synthesis. All three substitution-patterns were probed in combination with different benzylic modifications. These were primarily related to the lead **7** i.e. adenine and other nitrogen-containing

heterocycles as well as aromatics and more simple groups (e.g. alcohols, amines and nitriles). We herein report the initial results of this endeavour.

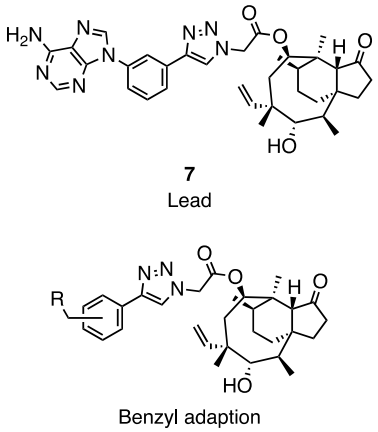


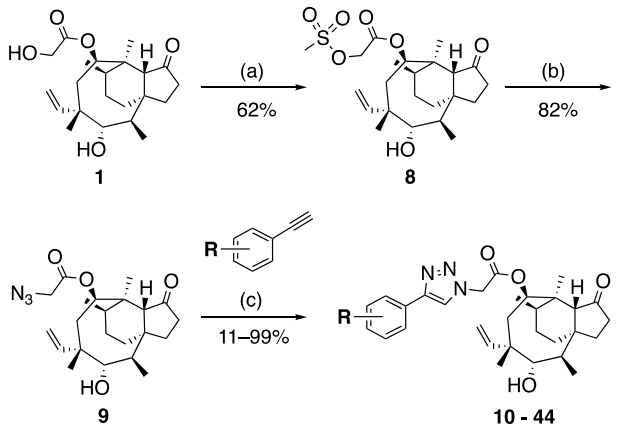
Figure 3. Structures of lead (compound **7**[14,19]) and the benzyl-adaption thereof.

Results and discussion

• Synthesis

The pleuromutilin conjugates were constructed by utilizing the same overall synthetic strategy as previously published (Scheme 1).[14,19,33] First, the glycolic ester of (+)-pleuromutilin **1** was activated by sulfonylation with mesyl chloride to grant **8**,[34] followed by substitution with sodium azide to form **9**, in moderate and good yields respectively. C22-functionalisation of **9** and the synthesis of **10**–**44** was achieved via the Cu(I)-catalysed alkyne-azide [3+2] cycloaddition (CuAAC) with yields ranging from very poor to excellent. The synthesis and derivatisation of the alkynes employed for the cycloaddition is summarized in Scheme 2 and 3 while the structures of the conjugates **10**–**44** are presented in Table 1.

Scheme 1.

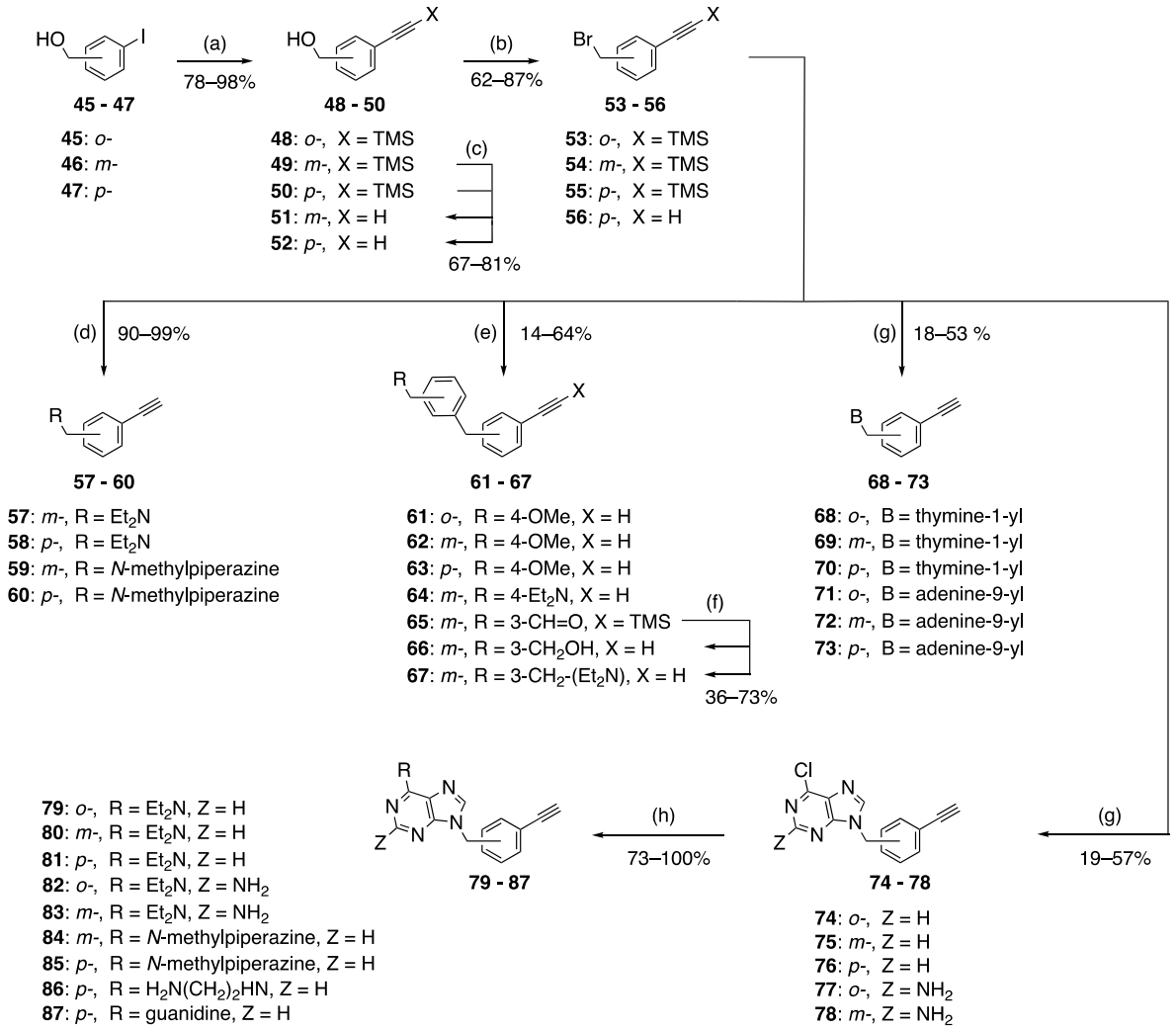


Reagents and conditions: (a) Mesyl-Cl, DCM, Et_3N, 0 °C → rt.; (b) NaN_3, acetone:H_2O, 70 °C; (c) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, t-BuOH:H_2O (1:1, v/v), 110 °C MW.

The strategy involved formation of the ethynylbenzyl scaffold initially by standard and efficient Sonogashira cross-coupling of iodobenzyl alcohols with trimethylsilylacetylene (**48**–**50**, Scheme 2). The benzylic position was then activated via bromination with PBr_3, which granted the benzylbromides **53**–**56** in good yields. From here on synthesis could easily diverge. The tertiary amines **57**–**60** were prepared by simple S_N2 substitutions in the presence of diethylamine or 1-

methylpiperazine in excellent yields. The benzylphenyl acetylenes **61**–**65** were synthesized via Suzuki cross-coupling with the appropriate boronic acids in poor to moderate yields. Subsequent reduction of **65** with $NaBH_4$ gave **66** in good yield, while reductive amination gave **67** in moderate yield.

Scheme 2.



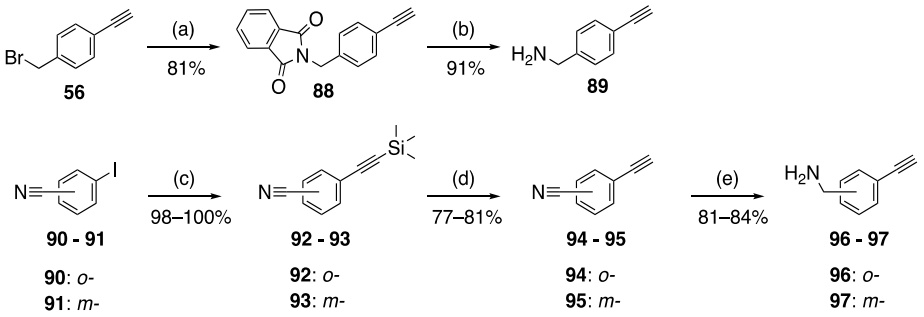
Reagents and conditions: (a) Trimethylsilylacetylene, $Pd(PPh_3)_4$, CuI, Anh. Piperidine 50 °C (**48**, **49**) or Anh. Et_3N at rt. (**50**). (b) DBU, PBr_3, DCM, 0 °C → rt.; (c) tetrabutylammonium fluoride (TBAF), THF, rt. (**51**) or K_2CO_3, MeOH, rt. (**52**); (d) Anh. THF, 55 °C, diethylamine (**57**, **58**) or 1-methylpiperazine **(59**, **60)** followed by K_2CO_3, MeOH, rt. (**57**, **59**); (e) Appropriate boronic acid, $Pd(Oac)_2$, PPh_3, K_3PO_4, tolune 80 °C (**61**) or $Pd(PPh_3)_4$, Na_2CO_3, THF:H_2O (2:1, v/v) (**62**–**65**) followed by K_2CO_3, MeOH:DCM 2:1 (v/v), rt. (**61**–**65**); (f) $NaBH_4$, MeOH, 0 °C → rt. (**66**) or diethylamine, $NaBH_4$, MeOH, 0 °C followed by K_2CO_3, MeOH:DCM 2:1 (v/v), rt. (**67**); (g) Appropriate nucleobase (B) or chloropurine, K_2CO_3, DMF, rt. (**68** – **72**, **74**, **77**, **78**) or NaH, DMF, rt. (**73**, **76**) followed by tetrabutylammonium fluoride (TBAF), THF, rt. (**69**, **70**, **75, 78**); (h) Appropriate amino species, Anh. EtOH, 75 °C (**79**–**86**) or i) guanidine·HCl, NaH, MeCN:DMF (2:1, v/v), rt. ii) 1,4-diazabicyclo[2.2.2]octane (DABCO), rt. (**87**)

Alkylation of the nucleobases (thymine, adenine, 6-chloropurine and 2-amino-chloropurine) was achieved in the presence of an inorganic base (K_2CO_3 or NaH) in DMF. The yields for **68**–**78** were mainly deteriorated by lack of regioselectivity, i.e. formation of the N3 isomer (thymines) or N7 isomer (purines), which in turn also complicated their subsequent separation during Flash Chromatography. The strategy was overall still more efficient and straightforward than our previous approach.

The chloropurins **74**–**78** were converted via nucleophilic aromatic substitution with various amines in anhydrous EtOH to grant **79**–**86**, generally in excellent yields. Compound **87** was prepared via guanidination in accordance with a procedure optimized by Česnek and coworkers.[35]

The primary benzylamines **89** and **96**–**97** were synthesized via two strategies (Scheme 3); the para analogue **89** via standard Gabriel synthesis, which proved highly efficient, simultaneously also granting the phthalimide **88**. The second strategy involved reduction of ethynylbenzonitriles with $LiAlH_4$,[36] which also proved efficient, granting the nitriles **94** and **95** as well.

Scheme 3.



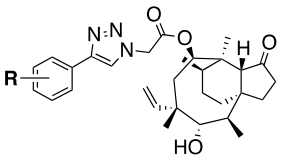
Reagents and conditions: (a) K-phthalimide, DMF, rt.; (b) $H_2N\text{-}NH_2 \cdot H_2O$ 50-60% w/w, EtOH, 78 °C; (a) Trimethylsilylacetylene, $Pd(PPh_3)_4$, CuI, Anh. Piperidine 80 °C; (d) K_2CO_3, MeOH, rt.; (e) i) 1 M $LiAlH_4$ in THF, THF, -10 °C → rt. ii) H_2O, NaOH (sat.).

- **MRSA susceptibility and SAR**

The antibacterial susceptibility (MIC) of conjugates **10**–**44** towards MRSA USA300 are listed in Table 1. It should be stressed that the correlation between *in-vitro* cell-based activity and raw binding affinity is indirect as additional barriers between the drug-like molecule and its target are introduced by the bacterial cell. The overall physicochemical properties of each compound thus also affect the observed MIC-value, where especially lipophilicity (often estimated by ClogP or PSA), solubility as well as solution and metabolic stability of the antibacterial, are significant.[37]

Baring no substituent, the phenyl **10** forms a natural baseline for comparison and discussion of the simple functionalizations incorporated in compounds **11**–**22**. The meta aniline **11** yielded a two-fold decrease in MIC over **10**, while the benzonitriles displayed reasonable activity with higher toleration of the ortho **12** than the equivalent meta **13**, which in turn displayed the same activity as **11**. Introduction of ionizability via the primary benzyl amines **14**–**16** resulted in a loss of activity over **10** and the analogous but neutral aniline **11**. Increasing lipophilicity by replacement of the NH_2-protons with ethyl-groups had no influence on activity between the meta analogues **15** and **17**, while a two-fold increase in MIC was observed between the para **16** and **18**. Despite the increased size, strain and ionizability of the piperazines **19** and **20**, the same activity and trend was observed (meta > para). Overall, the data clearly indicate that aliphatic, positively charged moieties are poorly accepted, and confers a loss in susceptibility over the bare phenyl **10** irrespective of ring position and may thus be a consequence of either poor permeation or stability. Although, as amines are present in the positive controls valnemulin **3** and retapamulin **4**, this suggests that the combination of amines and the relatively inflexible benzyl-triazole scaffold may not be able to accommodate a conformation that confers favourable electrostatic interactions.

Table 1. Minimal inhibitory concentration towards MRSA USA300



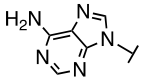
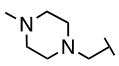
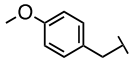
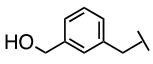
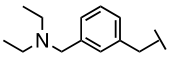
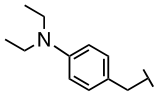
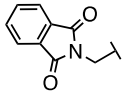
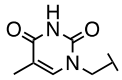
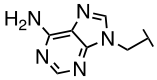
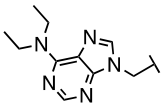
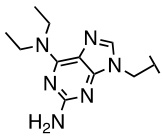
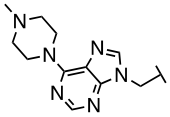
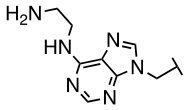
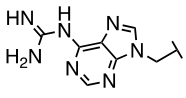
			MIC (USA300)[a,b]		Physicochemical descriptors		
Compound	R	Ring position	µg mL^{-1}	µM	MW [g mol^{-1}]	ClogP[c]	PSA[c]
3[d]	Valnemulin	-	0.06	0.10	601.3	5.56	119
4[d]	Retapamulin	-	0.25	0.48	517.8	5.21	67
7[e]		*m*-	0.06	0.094	638.7	3.19	158
7a[e]		*p*-	0.5	0.78	638.7	3.19	158
10		-	2	4.0	514.7	6.16	92
11		*m*-	1	1.9	520.7	4.94	135
12		*o*-	0.5	0.94	530.7	5.60	115
13		*m*-	1	1.9	530.7	5.60	115
14		*o*-	4	7.5	534.7	5.11	118
15		*m*-	2	3.7	534.7	5.11	118
16		*p*-	2	3.7	534.7	5.11	118
17		*m*-	2	3.4	590.8	7.05	95
18		*p*-	4	6.8	590.8	7.05	95
19		*m*-	2	3.2	617.8	6.35	98
20		*p*-	4	6.5	617.8	6.35	98
21		*m*-	0.5	0.94	535.7	5.12	112
22		*p*-	0.25	0.47	535.7	5.12	112
23		*o*-	2	25	625.8	7.85	101
24		*m*-	16	13	625.8	8.15	101
25		*p*-	8	1.6	625.8	8.15	101
26		*m*-	1	1.6	625.8	7.20	112
27		*m*-	2	2.9	680.9	9.12	95

Table 1. – continued.

			MIC (USA300) [a, b]		Physicochemical descriptors		
Compound	R	Ring position	µg mL^{-1}	µM	MW [g mol^{-1}]	ClogP[c]	PSA[c]
28		*m*-	8	12	666.9	9.45	95
29		*p*-	0.25	0.38	664.8	7.30	129
30		*o*-	1	1.6	643.8	4.79	141
31		*m*-	1	1.6	643.8	5.09	141
32		*p*-	0.13	0.20	643.8	5.09	141
33		*o*-	0.5	0.77	652.8	2.33	158
34		*m*-	0.25	0.38	652.8	2.63	158
35		*p*-	0.03	0.046	652.8	2.63	158
36		*o*-	1	1.4	708.9	4.78	135
37		*m*-	1	0.35	708.9	5.08	135
38		*p*-	0.25	0.68	708.9	5.08	135
39		*o*-	0.5	0.69	723.9	3.55	161
40		*m*-	0.5	0.69	723.9	3.85	161
41		*m*-	0.5	0.68	735.9	3.75	135
42		*p*-	0.25	0.34	735.9	3.75	138
43		*p*-	0.5	0.72	695.9	2.66	170
44		*p*-	0.5	0.72	695.8	2.23	194

[a] Minimal inhibitory concentration (MIC): lowest concentration of compound to fully inhibit visible growth of MRSA USA300

[b] MIC-values ≥ 0.5 µg mL^{-1} are n = 2, MIC-values ≤ 0.25 µg mL^{-1} are n = 3

[c] ChemDraw 18.2

[d] Positive control. For structures see Figure 1

[e] Dreier et al. (2014)[14]

The benzyl alcohols **21** and **22**, however, displayed good activity, with the para being two-fold better than its meta equivalent and comparable to that of retapamulin (0.25 μg mL^{-1}). Despite a presumably lower desolvation penalty, the alcohols and primary amines would likely accept and donate the same hydrogen bonds, which further suggest that the modest activity of **14**–**20** may be attributed to their ionizability. Overall, the incorporation of an additional phenyl-linker (**23**–**28**) did not enhance the antibacterial effect. While the activity of the ortho anisole **23** was equal to that of **10**, anisoles **24** and **25** were almost inactive. While the meta-positioned meta-benzyl alcohol **26** was accepted, it was less active than the alcohol **21**, possibly due to decreased solubility. The activity of the diethylamino **27** was moderate and in line with the trend observed for **14**–**20**. However, removal of the CH_2 and thereby ionizability resulted in a significant four-fold loss of activity for the diethylaniline **28**.

Introduction of the heterocycles of **29**–**44** proved more successful and in agreement with our previous work, as all displayed good antibacterial effect with several being pronounced. Unexpectedly, this included the para-phthalimide **29** despite its considerable lipophilicity – in fact, it was the only conjugate out of 12 with ClogP > 6 to go below 2 μg mL^{-1}. While both the ortho and meta thymines **30** and **31** were active, the para analogue **32** was more pronounced. This trend was repeated by the adenines **33**–**35** however to a greater extent as they were overall more active. Compound **35** proved to be exceptionally potent (MIC = 0.03 μg mL^{-1}), especially in comparison to the fairer activity of the ortho **33** and meta **34**. Again, to probe the influence of the NH_2-protons and increase lipophilicity this time on the more hydrophilic purine scaffold, the 6-amino was exchanged for the diethylamino-group of **36**–**38**. This was, however, poorly accepted across all three, with a significant 8-fold difference in MIC between **35** and **38**. The introduction of an amino-group in the 2-position retrieved some of the lost activity for the ortho **39** and meta **40** (two-fold).

Further extension of the C6 position and introduction of basic functional groups (**41**–**44**) were again poorly accepted. The additional structure-activity relationship (SAR) revealed that substitution with a methylpiperazine (**41**–**42**) granted potencies equal to the diethylamines **37** and **38**. Exchange with the analogous but more flexible ethylene diamine **43** yielded an additional 2-fold increase in MIC over the piperazine **42** and a significant 16-fold over **35**. Probing the effect of increased polarity and ability to donate hydrogen bonds by the guanidine **44**, it too was poorly tolerated. In summary, across the thymines, adenines and diethylaminopurines, a clear discrimination appears for both ring position (para >> meta > ortho) and nucleobase (adenine > thymine ≥ diethylaminopurine). Since one can assume almost identical in-group physicochemical properties, preference for the para-position is a result of increased multivalency and optimal positioning in the P-site of the PTC. Moreover, the thorough purine-C6 SAR evidently shows that independent of ring-position, substitution of the 6-amino group by aliphatic groups either cyclic or linear, neutral or basic is not accepted and translates to a significant loss of activity. The SAR further suggests that the 6-aminopurine-scaffold itself is able to occupy the available space, partake in hydrogen-bond donation but likely also form π-π interactions since some level of activity is retained after adenine modification.

For the time being, **35** is the only conjugate to display activity comparable to that of the lead **7**.[14] To investigate why, a simple 2D pharmacophore fingerprint model of **7** and **33**–**35** was prepared (Figure 4), with the displayed conformations having the highest degree of adenine overlap, as the experimental data suggest positioning and orientation of the nucleobase is the determining factor. Here it is evident that the C22-substituent of **35** can achieve the same overall topology as **7** and vice versa, whereas **34** and **33** cannot, with increasing dissimilarity furthermore being positively correlated with MIC. This may also explain why **7a**, the para-analogue of **7** displayed significantly

lower activity, as it is likewise unable to accommodate the correct positioning of the 6-amino group.

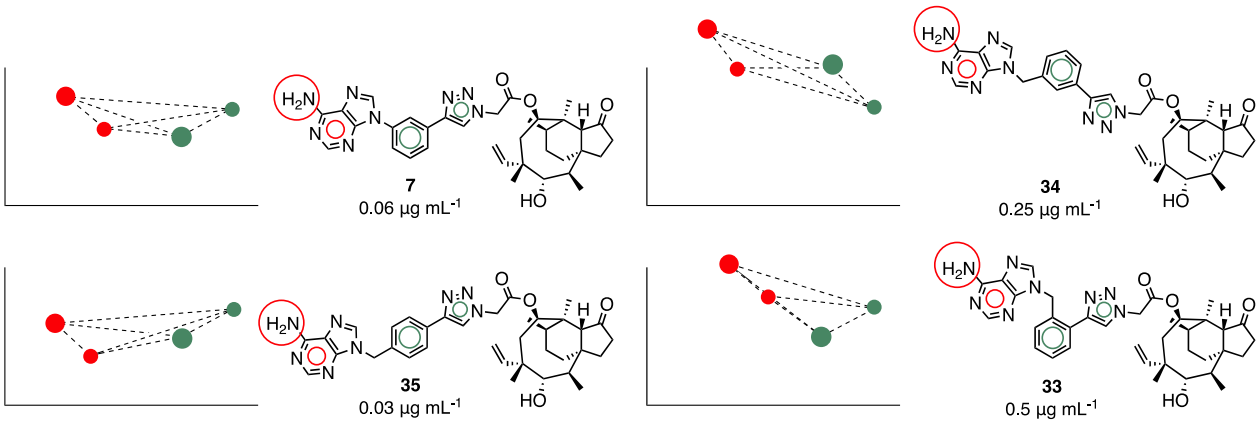


Figure 4. 2D Pharmacophore fingerprints of adenine-phenyltriazolo-pleuromutilin conjugates

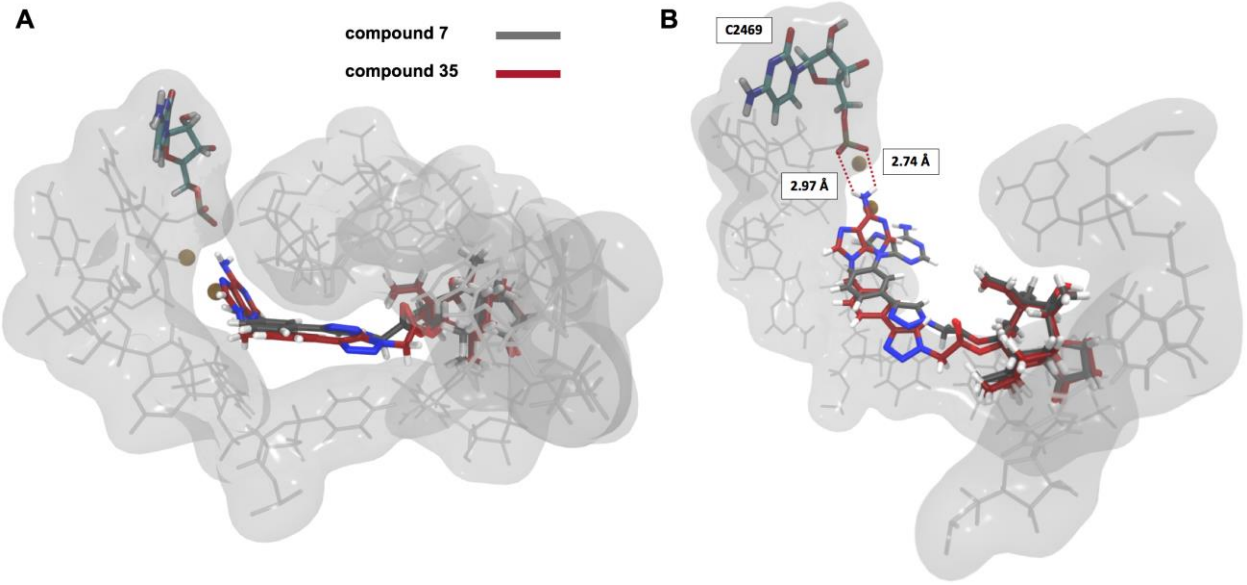


Figure 5. A: 3D superimposition of the lead **7** and **35** in the full PTC binding pocket (PDB: 5HL7). **B:** Top view of superimposition with nucleotides C2090, A2089 and G2088 removed to grant better overview of the potential hydrogen bonding between **35** and C2469 (S. *aureus* accession numbers).

The fingerprints of **7** and **35** were reproduced by a MM-GBSA model (0 Å flexibility), signified by the highly similar posing of the two conjugates (Figure 5), although in a more closed conformation than in Figure 4. In addition, compound **35** seems to more or less fully occupy the available space, as speculated earlier (Figure 5 A). Noteworthy is also the small but potentially crucial detail displayed in Figure 5 B, where the 6-amino of **35** has been simulated to be in close proximity of the backbone of C2469 (~ 3Å). As the 6-amino protons were identified as a clear pharmacophore in the SAR discussed earlier, this may, in fact, be due to their formation of strong anion-to-hydrogen bonding with the phosphate oxygens of C2469.

To our best knowledge, compound **35** is one of the most potent pleuromutilin conjugates against MRSA USA300 to be reported. To further verify its activity, compound **35** was screened against three additional clinical isolates of MRSA as well as a methicillin resistant *Staphylococcus Epidermis* isolate (MRSE, Table 2). High susceptibility of **35** was observed for all MRSA but also MRSE 933010, either outperforming or matching the activity of valnemulin (**3**). The activity of **35** against MRSA is thus likely to be on par with BC-7013 (**6**, Figure 1), especially if the difference in molecular weight is considered (~30%).

Table 2. Antibacterial activity of 35 against MRSA/MRSE

	MRSA USA300 [a,b]		MIC [μg mL^{-1}][a,b]			
Compound	MIC	MBC	MRSA 55508	MRSA 52518	MRSA 4828[c]	MRSE 933010
3[d]	0.06	0.12	0.12	0.06	0.06	0.25
4[d]	0.12	>2	0.25	0.12	0.25	0.5
35	0.03	0.12	0.06	0.03	0.06	0.25

[a] Minimal inhibitory concentration (MIC): lowest concentration of compound to fully inhibit visible growth of bacteria
Minimal bactericidal concentration (MBC): lowest concentration required to kill 99.9% of initial inoculum
[b] Values are $n = 3$
[c] Mupirocin resistant
[d] Positive control. For structure see Figure 1

The antibacterial mechanism of action against MRSA USA300 was also investigated and was probed via a minimum bactericidal concentration (MBC) assay (Table 2). As per convention,[37] an antibacterial is considered bacteriostatic if the MBC-to-MIC ratio is above 4 or bactericidal if equal to or below. As the latter is true for **35**, it classifies as a bactericidal agent against MRSA, which was also the case for valnemulin (**3**) furthermore in accordance with a study reported by Siricilla et al.[38]

To evaluate whether compound **35** can fare as a pre-clinical drug candidate, initial screening of mammalian *in vitro* cytotoxicity is key. This was therefore performed on four mammalian cell lines grown to confluency (Figure 6) where valnemulin (**3**) and retapamulin (**4**) were included for comparison and SDS as positive control. Noteworthy is the fact that no significant cytotoxicity was observed at any of investigated concentrations apart from a single point (valnemulin in PC-3). For this reason, only the diagrams which constitute the upper limit have been included in Figure 6. In fully differentiated human intestinal Caco-2 cells, compound **35**, valnemulin (**3**) and retapamulin (**4**) stimulated cellular ATP levels, whereas no stimulation took place in undifferentiated Caco-2 cells (Figure 6, A and B). Since no cellular necrosis was observed, even at the highest concentration (108 μg mL^{-1}), this suggest that **35** is not short-term cytotoxic towards Caco-2 cells. The increase in ATP levels in fully differentiated Caco-2 cells could be explained by an apoptotic element since it has been shown that increased ATP levels is a cellular event happing in cells entering an apoptotic phase.[39] However, this was only observed in fully differentiated Caco-2 cells, and not in undifferentiated Caco-2 cells or any of the other cell lines investigated. No short-term necrosis was observed in human prostate cancer cells (PC-3), human embryonic kidney cells expressing the organic anion transporter OAT3 (HEK-293 OAT3)[40] and canine renal

epithelial cells (MDCK I) (Figure 6, C, D and E), although a slight, yet significant, decrease in ATP levels was observed for valnemulin (**3**) in PC-3 cells at 100 µg mL^{-1} (22–23% reduction). The Caco-2 cell viability was also investigated using an MTT assay (Figure 6 F). In the concentration range investigated neither compound **35**, valnemulin (**3**) nor retapamulin (**4**) displayed any effects on cell viability in line with the CellTiter-Glo® results. A concentration dependent decrease in Caco-2 cell viability was however observed for the positive control SDS with an estimated IC_{50} of 147 µM (pIC_{50} = 3.83 ± 0.14).

In summary, it therefore seems reasonable to assume that compound **35** does not have an *in vitro* toxicity profile majorly different from two of the pleuromutilin conjugates approved for use in animals and humans, i.e valnemulin (**3**) and retapamulin (**4**), respectively.

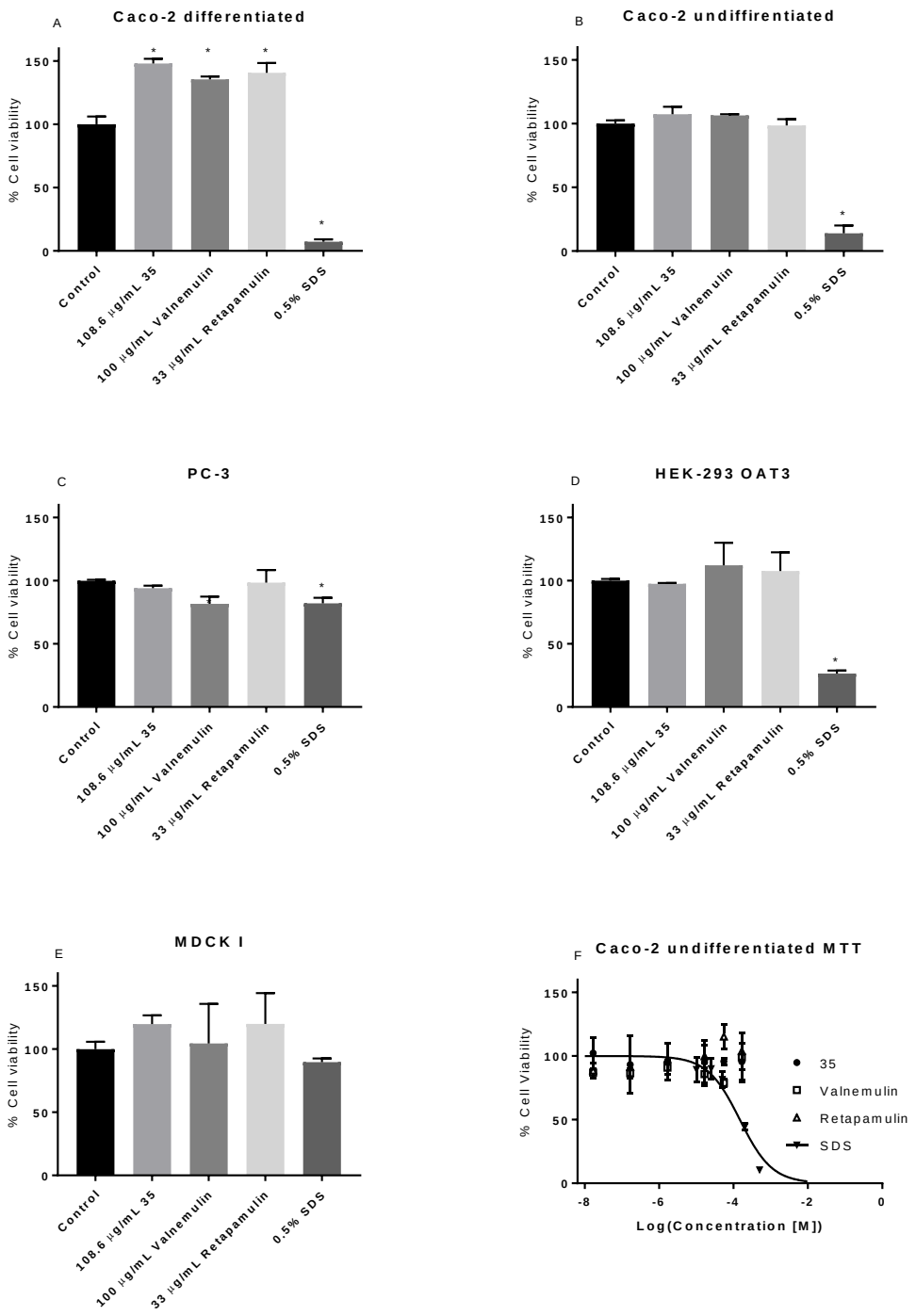


Figure 6. Effects of compound **35**, valnemulin (**3**), retapamulin (**4**) and SDS on cell viability. Cell viability was assessed via the CellTiter-Glo® 2.0 assay. ATP was measured in cells treated for 3 hours with either 10 mM HEPES in HBSS buffer (control) or different concentrations of antibacterial (μg/mL). **A**) Fully differentiated Caco-2 cells, (**B**) undifferentiated Caco-2 cells, (**C**) PC-3 cells (**D**) HEK-293 OAT3 cells, (**E**) MDCK I cells, and (**F**) MTT assay in undifferentiated Caco-2 cells. Fit obtained via GraphPad Prism 7.01. Each bar depicts the mean ± SD of replicates in 3 cell containing wells from one cell passage.

Conclusion

Through CuAAC functionalization of the powerful (+)-pleuromutilin (**1**) scaffold, a series of 35 new pleuromutilin conjugates were successfully designed, synthesized and subsequently screened on a MRSA USA300 *in vitro* susceptibility assay. This evaluation yielded several new structure-activity relationships and thereby provides an increased understanding of the pharmacophores associated with the benzyl-triazole-scaffold. Most notably is the clear preference for a para-substitution pattern of the benzyl system in combination with a nucleobase or modifications thereof. However, the introduction of a cationic moiety corresponded to a clear loss of *in vitro* activity, whether it be directly on the benzyl or its substituent.

Among the new pleuromutilin conjugates, the para-adenine **35** displayed excellent antibacterial activity against USA300 (MIC = 0.03 μg ml^{-1}, MBC = 0.12 μg ml^{-1}) but also three other isolates of MRSA as well as one MRSE. Adaption of the benzyl-triazole-scaffold thus proved successful as it achieved not only a two-fold increase in activity (USA300) but also a four-fold increase in total yield over the lead **7** (28% versus 7%). Extended SAR of the adenine C6 position revealed that the 6-amino is an essential pharmacophore. A MM-GBSA molecular model suggests that this is likely due to hydrogen bonding with the backbone of C2469.

As the scaffold of **35** is easy to modify, retains activity after modification and does not display significant levels of *in vitro* cytotoxicity, compound **35** or a derivative thereof may in the near future reveal itself as a highly promising drug-candidate for the fight against multi-resistant bacteria.

Experimental Section

General Methods

Commercially available solvents and starting materials were used unless otherwise stated. TLC was performed using silica gel 60 F254 plates and visualized at 254 nm or by staining with PMA, ninhydrin or $KMnO_4$ stains. For Flash Chromatography purification, silica gel 60 (0.040−0.063 mm, Merck) was used. ^1H and ^{13}C spectra were recorded at 400 and 101 MHz respectively, on a Bruker Avance III 400 at 300 K. Purity of all test compounds is > 95% and was determined by RPLC analysis performed using a Gemini C18 column (5 μm, 4.6 mm × 150 mm); flow, 1 mL/min; 10% MeCN in water (0 − 1 min), 10−100% MeCN in water (1 − 10 min), 100% MeCN (11 − 15 min), both solvents with 0.1% trifluoro acetic acid as modifier, UV detection at 254 nm.

General Procedure 1: CuAAC reaction

A small microwave-vial was charged with 22-azido-22-deoxypleuromutilin **9** (0.12–0.20 mmol), the appropriate alkyne (0.12–0.20 mmol), sodium ascorbate (0.025–0.040 mmol) and $CuSO_4{\cdot}5H_2O$ (0.012–0.020 mmol) before degassed t-BuOH:H_2O (1:1 v/v, 2.00–2.25 mL) was added. The vial was sealed and irradiated in a microwave reactor at 110 ^oC (normal/high absorption mode) for 30 min. The reaction mixture was concentrated *in vacuo* and the resulting residue was purified by Flash chromatography in either EtOAc:PE (0–100%), EtOAc:MeOH (0–20%) or MeOH:CH_2Cl_2 (0–20%) to afford the product as white crystals/foam.

22-[4-Phenyl-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (10) General Procedure 1 was applied with compound **9** (100.1 mg, 0.248 mmol), phenylacetylene (0.03 mL, 0.273 mmol), sodium ascorbate (4.8 mg, 0.024 mmol) and $CuSO_4{\cdot}5H_2O$ (5.8 mg, 0.023 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.6 mL). Flash Chromatography (MeOH:DCM, 10%). Yield: 104 mg (83%, 0.206 mmol); ^1H NMR (400 MHz, $CDCl_3$) δ 7.88–7.81 (m, 3H), 7.47–7.41 (m, 2H), 7.38–7.33 (m, 1H), 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.84 (d, *J* = 8.5 Hz, 1H), 5.35 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.22 (dd, *J* = 17.3, 1.5 Hz, 1H), 5.19–5.05 (m, 2H), 3.35 (dd, *J* = 10.7, 6.5 Hz, 1H), 2.32–2.06 (m, 5H), 1.80–1.38 (m, 8H), 1.36 (s, 3H), 1.33 (d, *J* = 16.3 Hz, 1H), 1.18 (s, 3H), 1.16–1.08 (m, 1H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.74 (d, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.6, 165.2, 148.5, 138.8, 130.5, 129.0, 128.5, 126.0, 121.0, 117.7, 74.7, 71.2, 58.1, 51.8, 45.5, 44.9, 44.2, 42.0,

36.7, 36.2, 34.5, 30.5, 26.9, 26.5, 24.9, 17.0, 14.8, 11.6; HRMS (ESI): m/z calculated for $C_{30}H_{39}N_8O_4$ ($M+H^+$) 506.3013 found 506.3003; HPLC purity at 254 nm: 100%

22-[4-(3-Aminophenyl)-1,2,3-triazol-1-yl)]-22-deoxypleuromutilin (11) General Procedure 1 was applied with compound **9** (90 mg, 0.22 mmol), 3-ethynylaniline (0.03 mL, 0.289 mmol), sodium ascorbate (4.6 mg, 0.023 mmol) and $CuSO_4 \cdot 5H_2O$ (6.1 mg, 0.024 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.5 mL). Flash Chromatography (MeOH:DCM, 10%). Yield: 93 mg (80%, 0.179 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (s, 1H), 7.25 (dd, *J* = 2.4, 1.5 Hz, 1H), 7.20 (t, *J* = 7.7 Hz, 1H), 7.13 (dt, *J* = 7.7, 1.3 Hz, 1H), 6.67 (ddd, *J* = 7.9, 2.4, 1.1 Hz, 1H), 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.82 (d, *J* = 8.5 Hz, 1H), 5.34 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.25–5.02 (m, 3H), 3.77 (s, 2H), 3.35 (dd, *J* = 10.2, 6.5 Hz, 1H), 2.33–2.05 (m, 6H), 1.80–1.58 (m, 4H), 1.54–1.24 (m, 8H), 1.18 (s, 3H), 1.16–1.07 (m, 1H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.72 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.7, 165.2, 148.6, 147.1, 138.8, 131.4, 129.9, 121.0, 117.7, 116.3, 115.2, 112.5, 74.7, 71.1, 58.1, 51.8, 45.5, 44.9, 44.2, 42.0, 36.7, 36.2, 34.5, 30.5, 26.9, 26.5, 24.9, 17.0, 14.8, 11.6; HRMS (ESI): m/z calculated for $C_{30}H_{40}N_4O_4$ ($M+H^+$) 521.3122 found 521.3129; HPLC purity at 254 nm: 99.6%

22-[4-(2-Cyanophenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (12) General Procedure 1 was applied with compound **9** (67 mg, 0.166 mmol), the alkyne **94** (21.1 mg, 0.166 mmol), sodium ascorbate (6.6 mg, 0.033 mmol) and $CuSO_4 \cdot 5H_2O$ (4.1 mg, 0.017 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.25 mL). Flash Chromatography (EtOAc:PE, 2.5–50%). Yield: 84 mg (96%, 0.158 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.48 (s, 1H), 8.39 (dt, *J* = 7.8, 1.0 Hz, 1H), 7.79–7.66 (m, 2H), 7.45 (td, *J* = 7.7, 1.2 Hz, 1H), 6.43 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.5 Hz, 1H), 5.35 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.26–5.10 (m, 3H), 3.35 (dd, *J* = 10.4, 6.5 Hz, 1H), 2.34–2.06 (m, 7H), 1.76 (dd, *J* = 14.5, 3.2 Hz, 1H), 1.72–1.59 (m, 2H), 1.58 (s, 2H), 1.55–1.36 (m, 5H), 1.34 (s, 4H), 1.19 (s, 3H), 1.12 (td, *J* = 14.0, 4.5 Hz, 1H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.74 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.5, 164.8, 144.1, 138.5, 133.6, 133.4, 128.3, 128.2, 123.7, 119.1, 117.7, 108.6, 74.6, 71.3, 58.0, 51.8, 45.4, 44.8, 44.0, 41.9, 36.6, 36.1, 34.4, 30.4, 26.8, 26.3, 24.8, 16.9, 14.6, 11.5; HRMS (ESI): m/z calculated for $C_{31}H_{39}N_4O_4$ ($M+H^+$) 531.2966 found 531.2640; HPLC purity at 254 nm: 99.5%

22-[4-(3-Cyanophenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (13) General Procedure 1 was applied with compound **9** (53 mg, 0.131 mmol), the alkyne **95** (16.6 mg, 0.131 mmol), sodium ascorbate (5.2 mg, 0.026 mmol) and $CuSO_4 \cdot 5H_2O$ (3.3 mg, 0.013 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.00 mL). Flash Chromatography (0–50% EtOAc:PE). Yield: 67 mg (96%, 0.126 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 (m, 2H), 7.95 (s, 1H), 7.64 (dt, *J* = 7.7, 1.4 Hz, 1H), 7.59–7.53 (m, 1H), 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.85 (d, *J* = 8.5 Hz, 1H), 5.35 (dd, *J* = 10.9, 1.5 Hz, 1H), 5.26–5.19 (m, 1H), 5.22–5.07 (m, 2H), 3.36 (dd, *J* = 10.6, 6.5 Hz, 1H), 2.35–2.02 (m, 7H), 1.83–1.06 (m, 22H), 0.87 (d, *J* = 0.8 Hz, 4H), 0.74 (d, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.5, 164.9, 146.2, 138.6, 131.7, 131.7, 129.9, 129.8, 129.3, 121.5, 118.5, 117.7, 113.2, 74.5, 71.3, 58.0, 51.7, 45.4, 44.8, 44.0, 41.9, 36.5, 36.1, 34.4, 30.3, 26.8, 26.4, 24.8, 16.9, 14.7, 11.5; HRMS (ESI): m/z calculated for $C_{31}H_{39}N_4O_4$ ($M+H^+$) 531.2966 found 531.2942; HPLC purity at 254 nm: 98.5%

22-[4-(2-(Aminomethyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (14) A slightly altered General Procedure 1 was applied with the alkyne **96** (20 mg, 0.153 mmol) as limiting reagent, compound **9** (65 mg, 0.161 mmol), tris((1-*tert*-butyl-1*H*-1,2,3-triazolyl)methyl)amine (TTTA, 6.9 mg, 0.016 mmol), sodium ascorbate (6.4 mg, 0.032 mmol) and $CuSO_4 \cdot 5H_2O$ (4.0 mg, 0.016 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.25 mL). Flash Chromatography (2–20% MeOH:DCM). Yield: 9 mg (11%, 0.017 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97 (br s, 1H), 7.53 (m, 2H), 7.40 (m, 2H), 6.43 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.85 (d, *J* = 8.5 Hz, 1H), 5.39 – 5.33 (m, 1H), 5.26 – 5.08 (m, 3H), 3.56 (s, 2H), 3.37 (d, *J* = 6.4 Hz, 1H), 2.33 – 2.07 (m, 6H), 1.78 (d, *J* = 14.4 Hz, 1H), 1.74 – 1.60 (m, 3H), 1.58 – 1.40 (m, 3H), 1.38 (s, 3H), 1.33 (s, 1H), 1.25 (s, 1H), 1.19 (s, 3H), 1.13 (dt, *J* = 13.4, 6.7 Hz, 1H), 0.88 (d, *J* = 7.0 Hz, 3H), 0.74 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.6, 165.0, 138.8, 129.4, 117.8, 74.7, 71.4, 58.1, 45.6, 44.9, 44.2, 42.0, 36.7, 36.2, 34.5, 30.5, 29.8, 27.0, 26.6, 25.0, 17.0, 14.8, 11.6; HRMS (ESI): m/z calculated for $C_{31}H_{43}N_4O_4$ ($M+H^+$) 535.3279 found 535.3260; HPLC purity at 254 nm: 96.8%

22-[4-(3-(Aminomethyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (15) A slightly altered General Procedure 1 was applied with the alkyne **97** (20 mg, 78% m/m, 0.118 mmol) as limiting reagent, compound **9** (50 mg, 0.124 mmol), sodium ascorbate (4.9 mg, 0.025 mmol) and $CuSO_4 \cdot 5H_2O$ (3.0 mg, 0.012 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.00 mL). Mixture

evaporated onto Celite. Flash Chromatography (2–20% MeOH : CH_2Cl_2). Yield: 26 mg (42%, 0.049 mmol); ^{1}H NMR (400 MHz, DMSO) δ 8.50 (s, 1H), 7.87 (s, 1H), 7.71 (d, *J* = 7.7 Hz, 1H), 7.39 (d, *J* = 7.6 Hz, 1H), 7.35–7.30 (m, 1H), 6.14 (dd, *J* = 17.8, 11.2 Hz, 1H), 5.59 (d, *J* = 8.3 Hz, 1H), 5.47–5.27 (m, 2H), 5.17–5.01 (m, 2H), 4.56 (d, *J* = 5.9 Hz, 1H), 3.83 (s, 2H), 3.44–3.40 (m, 2H), 2.41 (s, 1H), 2.24–1.98 (m, 4H), 1.70–1.26 (m, 7H), 1.24 (s, 3H), 1.08 (s, 3H), 0.81 (d, *J* = 6.9 Hz, 3H), 0.65 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 217.0, 165.6, 146.5, 146.5, 140.7, 130.4, 128.7, 126.9, 124.1, 123.5, 122.7, 115.4, 72.5, 70.6, 57.1, 51.3, 44.9, 44.1, 43.3, 41.5, 36.4, 36.2, 33.9, 30.0, 28.5, 26.5, 24.4, 16.1, 14.2, 11.5; HRMS (ESI): m/z calculated for $C_{31}H_{43}N_4O_4$ ($M+H^+$) 535.3279 found 535.3289; HPLC purity at 254 nm: 96.8%

22-[4-(4-(Aminomethyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (16) A slightly altered General Procedure 1 was applied with the alkyne **89** (20 mg, 0.153 mmol) as limiting reagent, compound **9** (50 mg, 0.161 mmol), sodium ascorbate (6.4 mg, 0.032 mmol) and $CuSO_4{\cdot}5H_2O$ (4.0 mg, 0.016 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.25 mL). Flash Chromatography (PE : EtOAc : MeOH, 1:1:0 → 0:9:1 → 0:4:1). Yield: 51 mg (62%, 0.095 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (s, 1H), 7.81 (d, *J* = 7.5 Hz, 2H), 7.39 (d, *J* = 7.8 Hz, 2H), 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.4 Hz, 1H), 5.34 (dd, *J* = 11.0, 1.4 Hz, 1H), 5.23 (d, *J* = 1.5 Hz, 1H), 5.19 – 5.05 (m, 2H), 4.15 (s, 3H), 3.36 (d, *J* = 6.4 Hz, 1H), 2.34 – 1.96 (m, 9H), 1.82 – 1.38 (m, 7H), 1.35 (s, 4H), 1.32 – 1.23 (m, 2H), 1.18 (s, 3H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.73 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.5, 165.1, 148.0, 138.6, 129.5, 128.1, 126.2, 120.9, 118.8, 117.6, 77.2, 74.6, 71.1, 58.0, 51.7, 45.4, 44.8, 44.0, 41.9, 36.6, 36.1, 34.4, 30.4, 26.8, 26.4, 24.8, 16.8, 14.6, 11.5; HRMS (ESI): m/z calculated for $C_{31}H_{43}N_4O_4$ ($M+H^+$) 535.3279 found 535.3268; HPLC purity at 254 nm: 96.3%

22-[4-(3-((Diethylamino)methyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (17) A slightly altered General Procedure 1 was applied with the alkyne **57** (28 mg, 0.149 mmol) as limiting reagent, compound **9** (65 mg, 0.161 mmol), sodium ascorbate (6.3 mg, 0.032 mmol) and $CuSO_4{\cdot}5H_2O$ (4.0 mg, 0.016 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.25 mL). Flash Chromatography (PE : EtOAc : MeOH, 1:4:0 → 0:1:0 → 0:9:1 → 0:6:1). Yield: 81 mg (92%, 0.137 mmol,); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (s, 1H), 7.82 (s, 1H), 7.74 (dt, *J* = 7.3, 1.7 Hz, 1H), 7.41–7.32 (m, 2H), 6.43 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.84 (d, *J* = 8.5 Hz, 1H), 5.35 (dd, *J* =

11.0, 1.5 Hz, 1H), 5.22 (dd, *J* = 17.5, 1.5 Hz, 1H), 5.12 (q, *J* = 17.5 Hz, 2H), 3.66 (s, 2H), 3.36 (d, *J* = 6.4 Hz, 1H), 2.59 (q, *J* = 7.1 Hz, 4H), 2.35–2.03 (m, 6H), 1.77 (dt, *J* = 14.5, 3.1 Hz, 1H), 1.72–1.62 (m, 2H), 1.62–1.39 (m, 4H), 1.37 (s, 4H), 1.18 (s, 3H), 1.08 (t, *J* = 7.1 Hz, 6H), 0.88 (d, *J* = 7.0 Hz, 3H), 0.74 (d, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.5, 165.1, 148.5, 138.6, 130.2, 129.1, 128.8, 126.5, 124.5, 121.0, 117.6, 74.6, 71.0, 58.0, 57.4, 51.7, 46.7, 45.4, 44.8, 44.0, 41.9, 36.6, 36.1, 34.4, 30.4, 26.8, 26.4, 24.8, 16.9, 14.7, 11.5, 11.5; HRMS (ESI): m/z calculated for $C_{35}H_{51}N_4O_4$ ($M+H^+$) 591.3905 found 591.3896; HPLC purity at 254 nm: 98.7%

22-[4-(4-((Diethylamino)methyl)phenyl)-1*H*-1,2,3-triazol-1-yl-22-deoxypleuromutilin (18) General Procedure 1 was applied with compound **9** (60 mg, 0.149 mmol), the alkyne **58** (27.9 mg, 0.149 mmol), sodium ascorbate (5.9 mg, 0.030 mmol) and $CuSO_4{\cdot}5H_2O$ (3.0 mg, 0.015 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.25 mL). Flash Chromatography (MeOH:EtOAc, 0% → 1% → 5% → 10%). Yield: 80 mg (91%, 0.135 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (s, 1H), 7.79 (d, *J* = 8.2 Hz, 2H), 7.43 (d, *J* = 8.2 Hz, 2H), 6.43 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.5 Hz, 1H), 5.35 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.22 (dd, *J* = 17.4, 1.5 Hz, 1H), 5.19 – 5.05 (m, 2H), 3.69 (s, 2H), 3.35 (d, *J* = 6.5 Hz, 1H), 2.62 (q, *J* = 7.1 Hz, 4H), 2.34 – 2.06 (m, 5H), 2.05 (d, *J* = 1.4 Hz, 1H), 1.82 – 1.39 (m, 6H), 1.36 (d, *J* = 1.9 Hz, 4H), 1.18 (s, 3H), 1.10 (t, *J* = 7.1 Hz, 6H), 0.88 (d, *J* = 7.0 Hz, 3H), 0.73 (d, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.5, 165.1, 148.3, 138.6, 129.7, 129.2, 125.8, 120.7, 117.6, 77.2, 74.6, 71.1, 58.0, 57.0, 51.7, 46.5, 45.4, 44.8, 44.0, 41.9, 36.6, 36.1, 34.4, 30.4, 26.8, 26.4, 24.8, 16.8, 14.7, 11.5, 11.3; HRMS (ESI): m/z calculated for $C_{35}H_{51}N_4O_4$ ($M+H^+$) 591.3905 found 591.3893; HPLC purity at 254 nm: 96.3%

22-[4-(3-((4-Methylpiperazin-1-yl)methyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (19) A slightly altered General Procedure 1 was applied with the alkyne **59** (32.8 mg, 0.153 mmol) as limiting reagent, compound **9** (65 mg, 0.161 mmol), sodium ascorbate (6.4 mg, 0.032 mmol) and $CuSO_4{\cdot}5H_2O$ 4.0 mg, 0.016 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.25 mL). Flash Chromatography (MeOH:DCM, 2–15%). Yield: 65 mg (69%, 0.105 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (s, 1H), 7.80 (s, 1H), 7.73 (dt, *J* = 7.5, 1.5 Hz, 1H), 7.37 (d, *J* = 7.5 Hz, 1H), 7.35–7.29 (m, 1H), 6.43 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.84 (d, *J* = 8.5 Hz, 1H), 5.35 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.22 (dd, *J* = 17.4, 1.5 Hz, 1H), 5.12 (q, *J* = 17.5 Hz, 2H), 3.56 (s, 2H), 3.36 (s, 1H), 2.51 (s, 8H), 2.35–2.06 (m, 9H), 1.77 (dt, *J* = 14.6, 3.2 Hz, 1H), 1.73–1.39 (m, 6H),

1.37 (s, 3H), 1.34 (d, *J* = 16.4 Hz, 1H), 0.88 (d, *J* = 7.0 Hz, 3H), 0.74 (d, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.5, 165.1, 148.4, 139.0, 138.6, 130.3, 129.2, 128.8, 126.6, 124.7, 120.9, 117.6, 77.2, 74.6, 71.1, 62.9, 58.0, 55.1, 53.0, 51.7, 46.0, 45.4, 44.8, 44.1, 41.9, 36.6, 36.1, 34.4, 30.4, 26.8, 26.4, 24.8, 16.9, 14.7, 11.5; HRMS (ESI): m/z calculated for $C_{36}H_{52}N_5O_4$ ($M+H^+$) 618.4014 found 618.4002; HPLC purity at 254 nm: 99.5%

22-[4-(4-((4-Methylpiperazin-1-yl)methyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (20) A slightly altered General Procedure 1 was applied with the alkyne **60** (32.8 mg, 0.153 mmol) as limiting reagent, compound **9** (65 mg, 0.161 mmol), sodium ascorbate (6.4 mg, 0.032 mmol) and $CuSO_4 \cdot 5H_2O$ 4.0 mg, 0.016 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.25 mL). Flash Chromatography (MeOH:DCM, 2–15%). Yield: 82 mg (87%, 0.133 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (s, 1H), 7.78 (d, *J* = 8.2 Hz, 2H), 7.39 (d, *J* = 8.3 Hz, 2H), 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.5 Hz, 1H), 5.35 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.22 (dd, *J* = 17.4, 1.5 Hz, 1H), 5.19–5.04 (m, 2H), 3.54 (s, 2H), 3.34 (d, *J* = 7.4 Hz, 1H), 2.50 (s, 8H), 2.31 (s, 3H), 2.31–2.04 (m, 6H), 1.77 (dd, *J* = 14.5, 3.1 Hz, 1H), 1.72–1.39 (m, 6H), 1.35 (s, 3H), 1.31 (s, 1H), 1.18 (s, 3H), 1.17–1.08 (m, 1H), 0.88 (d, *J* = 7.0 Hz, 3H), 0.73 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.5, 165.1, 148.3, 138.6, 138.6, 129.7, 129.2, 125.8, 120.7, 117.6, 77.2, 74.6, 71.1, 62.7, 58.0, 55.1, 53.0, 51.7, 46.0, 45.4, 44.8, 44.0, 41.9, 36.6, 36.1, 34.4, 30.4, 26.8, 26.4, 24.8, 16.8, 14.7, 11.5; HRMS (ESI): m/z calculated for $C_{36}H_{52}N_5O_4$ ($M+H^+$) 618.4014 found 618.4025, HPLC purity at 254 nm: 95.6%.

22-[4-(3-(Hydroxymethyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (21) General Procedure 1 was applied with compound **9** (81 mg, 0.201 mmol), the alkyne **51** (26.7 mg, 0.201 mmol), sodium ascorbate (5.8 mg, 0.029 mmol) and $CuSO_4 \cdot 5H_2O$ (5.0 mg, 0.020 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.25 mL). Flash Chromatography (MeOH : EtOAc, 0 → 80%). Yield: 82 mg (76%, 0.153 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (s, 1H), 7.87–7.85 (m, 1H), 7.77 (dt, *J* = 7.7, 1.5 Hz, 1H), 7.44 (t, *J* = 7.6 Hz, 1H), 7.39–7.34 (m, 1H), 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.84 (d, *J* = 8.5 Hz, 1H), 5.35 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.22 (dd, *J* = 17.4, 1.5 Hz, 1H), 5.12 (q, *J* = 17.5 Hz, 2H), 4.77 (d, *J* = 5.5 Hz, 2H), 3.35 (dd, *J* = 10.6, 6.5 Hz, 1H), 2.34–2.18 (m, 4H), 2.16–2.07 (m, 2H), 1.81–1.59 (m, 3H), 1.56 (s, 2H), 1.55–1.39 (m, 2H), 1.36 (s, 4H), 1.20–1.08 (m, 4H), 0.88 (d, *J* = 7.1 Hz, 3H), 0.74 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ

216.5, 165.1, 148.2, 141.7, 138.6, 130.6, 129.2, 126.9, 125.1, 124.4, 120.9, 117.7, 74.6, 71.1, 65.2, 58.0, 51.7, 45.4, 44.8, 44.1, 41.9, 36.6, 36.1, 34.4, 30.4, 26.8, 26.4, 24.8, 16.9, 14.7, 11.5; HRMS (ESI): m/z calculated for $C_{31}H_{42}N_3O_5$ ($M+H^+$) 536.3119 found 536.3100; HPLC purity at 254 nm: 96.7%

22-[4-(4-(Hydroxymethyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (22) General Procedure 1 was applied with compound **9** (80 mg, 0.198 mmol), the alkyne **52** (26.2 mg, 0.198 mmol), sodium ascorbate (7.9 mg, 0.040 mmol) and $CuSO_4 \cdot 5H_2O$ (5.0 mg, 0.020 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.25 mL). Flash Chromatography (15–100% EtOAc:PE). Yield: 102 mg (96%, 0.191 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (s, 1H), 7.85 – 7.80 (m, 2H), 7.47 – 7.41 (m, 2H), 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.84 (d, *J* = 8.5 Hz, 1H), 5.35 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.22 (dd, *J* = 17.3, 1.5 Hz, 1H), 5.19 – 5.04 (m, 2H), 4.74 (s, 2H), 3.40 – 3.31 (m, 1H), 2.33 – 2.04 (m, 5H), 1.77 (dq, *J* = 14.4, 3.1 Hz, 1H), 1.66 (tdd, *J* = 13.8, 10.6, 6.6 Hz, 3H), 1.58 – 1.39 (m, 4H), 1.36 (s, 4H), 1.18 (s, 3H), 1.13 (td, *J* = 14.2, 4.7 Hz, 4H), 0.88 (d, *J* = 7.0 Hz, 3H), 0.73 (d, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.5, 165.1, 148.1, 141.1, 138.6, 129.7, 127.5, 126.0, 120.8, 117.6, 74.6, 71.1, 65.1, 58.0, 51.7, 45.4, 44.8, 44.0, 41.9, 36.6, 36.1, 34.4, 30.4, 26.8, 26.4, 24.8, 16.8, 14.6, 11.5; HRMS (ESI): m/z calculated for $C_{31}H_{42}N_3O_5$ ($M+H^+$) 536.3119 found 536.3100 (App. 13.C); HPLC purity at 254 nm: 96.6%

22-[4-(2-(4-Methoxybenzyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (23) General Procedure 1 was applied with compound **9** (81 mg, 0.201 mmol), the alkyne **61** (44 mg, 0.201 mmol), sodium ascorbate (3.7 mg, 0.015 mmol) and $CuSO_4 \cdot 5H_2O$ (5.0 mg, 0.025 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.5 mL). Flash Chromatography (MeOH:CH_2Cl_2, 0% → 1% → 2%). Yield: 56.4 mg (45%, 0.090 mmol); ^{1}H NMR (400 MHz, DMSO) δ 7.74–7.68 (m, 1H), 7.45 (s, 1H), 7.36–7.30 (m, 2H), 7.24–7.19 (m, 1H), 7.02–6.96 (m, 2H), 6.82–6.76 (m, 2H), 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.81 (d, *J* = 8.5 Hz, 1H), 5.34 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.21 (dd, *J* = 17.4, 1.5 Hz, 1H), 5.13–4.99 (m, 2H), 4.11 (s, 2H), 3.76 (s, 3H), 2.31–2.05 (m, 6H), 1.80–1.61 (m, 4H), 1.54–1.34 (m, 6H), 1.32 (s, 3H), 1.17 (s, 3H), 0.88 (d, *J* = 7.0 Hz, 3H), 0.67 (d, *J* = 7.1 Hz, 3H). ^{13}C NMR (DMSO, 101 MHz) δ 211.2, 159.7, 152.7, 142.0, 133.4, 127.7, 125.6, 124.6, 124.4, 123.4, 121.3, 117.9, 112.3, 108.7, 69.3, 65.6, 52.9, 52.7, 50.0, 46.3, 40.2, 39.5, 38.8, 36.6, 33.2,

31.3, 30.8, 29.1, 25.1, 21.5, 21.1, 19.6, 11.5, 9.4; HRMS (ES) m/z calculated for $C_{38}H_{48}N_3O_5$ ($M+H^+$) 626.3594 found 626.3575; HPLC purity at 254 nm: 96.7%

22-[4-(3-(4-Methoxybenzyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (24)

General Procedure 1 was applied with compound **9** (81 mg, 0.201 mmol), the alkyne **62** (44 mg, 0.201 mmol), sodium ascorbate (3.7 mg, 0.015 mmol) and $CuSO_4 \cdot 5H_2O$ (5.0 mg, 0.025 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.5 mL). Flash Chromatography (MeOH:CH_2Cl_2, 0% → 1% → 2%). Yield: 111 mg (88%, 0.177 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (s, 1H), 7.69 (d, *J* = 1.8 Hz, 1H), 7.67–7.62 (m, 1H), 7.34 (t, *J* = 7.6 Hz, 1H), 7.19–7.10 (m, 3H), 6.88–6.80 (m, 2H), 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.5 Hz, 1H), 5.34 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.21 (dd, *J* = 17.4, 1.5 Hz, 1H), 5.10 (q, *J* = 17.5 Hz, 2H), 3.97 (s, 2H), 3.78 (s, 3H), 3.35 (d, *J* = 6.5 Hz, 1H), 2.92 (d, *J* = 5.6 Hz, 2H), 2.39 (s, 2H), 2.33–2.04 (m, 8H), 1.80–1.59 (m, 6H), 1.54–1.25 (m, 9H), 1.18 (s, 3H), 1.19–1.06 (m, 5H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.73 (d, *J* = 7.0 Hz, 3H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 216.5, 165.1, 158.1, 148.4, 142.3, 138.6, 133.0, 130.4, 129.9, 129.0, 128.9, 126.3, 123.6, 120.9, 117.6, 114.0, 74.5, 71.0, 58.0, 55.3, 51.6, 46.7, 45.4, 44.8, 44.0, 41.9, 41.0, 36.6, 36.1, 34.4, 30.4, 26.8, 26.4, 24.8, 16.8, 14.6, 11.4; HRMS (ESI) m/z calculated for $C_{38}H_{48}N_3O_5$ ($M+H^+$) 626.3594 found 626.3545; HPLC purity at 254 nm: 97.7%

22-[4-(4-(4-Methoxybenzyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (25)

General Procedure 1 was applied with compound **9** (50 mg, 0.124 mmol), the alkyne **63** (27 mg, 0.124 mmol), sodium ascorbate (2.2 mg, 0.013 mmol) and $CuSO_4 \cdot 5H_2O$ (3.3 mg, 0.013 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.5 mL). Flash Chromatography (MeOH:CH_2Cl_2, 0% → 1% → 2% → 5%). Yield: 69 mg (84%, 0.110 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (s, 1H), 7.73 (d, *J* = 8.2 Hz, 2H), 7.23 (d, *J* = 8.2 Hz, 2H), 7.12 (d, *J* = 8.6 Hz, 2H), 6.84 (d, *J* = 8.7 Hz, 2H), 6.41 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.81 (d, *J* = 8.5 Hz, 1H), 5.33 (dd, *J* = 10.9, 1.5 Hz, 1H), 5.20 (dd, *J* = 17.4, 1.5 Hz, 1H), 5.16–5.01 (m, 2H), 3.94 (s, 2H), 3.78 (s, 3H), 3.41–3.30 (m, 1H), 2.33–2.03 (m, 6H), 1.79–1.37 (m, 8H), 1.34 (s, 5H), 1.17 (s, 3H), 1.14–1.05 (m, 1H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.71 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.6, 165.1, 158.1, 148.2, 141.9, 138.7, 133.0, 129.9, 129.3, 128.1, 126.0, 120.7, 117.5, 114.0, 74.5, 71.0, 58.0, 55.3, 51.6, 45.4, 44.7, 44.0, 41.9, 40.8, 36.6, 36.1, 34.4, 30.3, 26.8, 26.5, 24.8, 16.8, 11.5; HRMS (ESI) m/z calculated for $C_{38}H_{48}N_3O_5$ ($M+H^+$) 626.8180 found 626.3560; HPLC purity at 254 nm: 99.2%

22-[4-(3-(3-(Hydroxymethyl)benzyl)phenyl)-1H-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (26) General Procedure 1 was applied with compound **9** (81 mg, 0.201 mmol), the alkyne **66** (44 mg, 0.201 mmol), sodium ascorbate (4.0 mg, 0.020 mmol) and $CuSO_4 \cdot 5H_2O$ (5.0 mg, 0.020 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.5 mL). Flash Chromatography (MeOH:CH_2Cl_2, 0% → 1% → 2% → 5%). Yield: 32 mg (26%, 0.051 mmol); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.82 (s, 1H), 7.70 (s, 1H), 7.65 (dd, *J* = 7.8, 1.4 Hz, 1H), 7.35 (t, *J* = 7.7 Hz, 1H), 7.28 (d, *J* = 8.0 Hz, 1H), 7.24–7.13 (m, 4H), 6.41 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.82 (d, *J* = 8.5 Hz, 1H), 5.34 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.21 (dd, *J* = 17.4, 1.5 Hz, 1H), 5.17–5.02 (m, 2H), 4.66 (s, 2H), 4.03 (s, 2H), 3.34 (d, *J* = 8.1 Hz, 1H), 2.32–2.03 (m, 6H), 1.81–1.57 (m, 7H), 1.45 (m, 4H), 1.34 (s, 3H), 1.32–1.25 (m, 2H), 1.17 (s, 3H), 1.12 (d, *J* = 4.5 Hz, 1H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.72 (d, *J* = 7.0 Hz, 3H).^{13}C-NMR (101 MHz, $CDCl_3$) δ 216.6, 165.1, 148.3, 141.7, 141.2, 138.6, 130.5, 129.1, 128.8, 128.3, 127.5, 126.5, 124.9, 123.8, 120.9, 117.6, 74.5, 71.0, 65.3, 58.0, 51.6, 45.4, 44.7, 44.0, 41.9, 41.9, 36.6, 36.1, 34.4, 30.3, 26.8, 26.4, 24.8, 16.8, 14.6, 11.4.; HRMS (ESI) m/z calculated for $C_{38}H_{48}N_3O_5$ ($M+H^+$) 626.3594 found 626.3568; HPLC purity at 254 nm: 95.7%

22-[4-(3-(3-(*N*,*N*-Diethylaminomethyl)benzyl)phenyl)-1H-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (27) General Procedure 1 was applied with compound **9** (40 mg, 0.10 mmol), the alkyne **67** (27 mg, 0.10 mmol), sodium ascorbate (1.7 mg, 0.010 mmol) and $CuSO_4 \cdot 5H_2O$ (2.5 mg, 0.010 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.5 mL). Flash Chromatography (MeOH:CH_2Cl_2, 0% → 1% → 2% → 5%). Yield: 55 mg (81%, 0.081 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (s, 1H), 7.71 (s, 1H), 7.65 (dd, *J* = 7.8, 1.4 Hz, 1H), 7.34 (t, *J* = 7.7 Hz, 1H), 7.25–7.15 (m, 4H), 7.08 (dt, *J* = 6.7, 1.7 Hz, 1H), 6.41 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.4 Hz, 1H), 5.33 (dd, *J* = 10.9, 1.5 Hz, 1H), 5.21 (dd, *J* = 17.4, 1.5 Hz, 1H), 5.18–5.02 (m, 2H), 4.02 (s, 2H), 3.56 (s, 2H), 3.35 (t, *J* = 7.3 Hz, 1H), 2.53 (m, 4H), 2.31–2.06 (m, 6H), 1.81–1.39 (m, 8H), 1.38–1.24 (m, 5H), 1.18 (s, 3H), 1.04 (t, *J* = 7.2 Hz, 6H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.73 (d, *J* = 7.0 Hz, 3H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 216.6, 165.1, 148.4, 142.0, 140.7, 138.6, 130.4, 129.6, 129.0, 129.0, 128.4, 127.5, 126.9, 126.5, 123.7, 120.9, 117.6, 74.5, 71.0, 58.0, 57.4, 51.6, 46.7, 45.4, 44.8, 44.0, 41.9, 36.6, 36.1, 34.4, 30.4, 26.8, 26.4, 24.8, 16.8, 14.7, 11.6, 11.5jjffv; HRMS (ESI) m/z calculated for $C_{42}H_{57}N_4O_4$ ($M+H^+$) 681.4380 found 681.6373; HPLC purity for 254 nm: 95.6%

22-[4-(3-((4-*N*,*N*-Diethylamino)benzyl)phenyl)-1H-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (28) General Procedure 1 was applied with compound **9** (81 mg, 0.201 mmol), the alkyne **64** (53 mg, 0.201 mmol), sodium ascorbate (2.5 mg, 0.020 mmol) and $CuSO_4 \cdot 5H_2O$ (5.0 mg, 0.020 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.5 mL). Flash Chromatography (MeOH:CH_2Cl_2, 0% → 1% → 2% → 5%). Yield: 95 mg (71%, 0.142 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (s, 1H), 7.70 (d, *J* = 1.8 Hz, 1H), 7.64 (s, 1H), 7.33 (t, *J* = 7.7 Hz, 1H), 7.18 (dt, *J* = 7.8, 1.4 Hz, 1H), 7.06 (d, *J* = 8.2 Hz, 2H), 6.63 (d, *J* = 8.2 Hz, 2H), 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.5 Hz, 1H), 5.34 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.30 (s, 2H), 5.21 (dd, *J* = 17.4, 1.5 Hz, 1H), 5.10 (q, *J* = 17.5 Hz, 2H), 3.92 (s, 2H), 3.32 (q, *J* = 7.2 Hz, 4H), 2.35–2.04 (m, 6H), 1.81–1.58 (m, 4H), 1.57–1.44 (m, 3H), 1.43–1.39 (m, 1H), 1.36 (s, 4H), 1.28 (d, *J* = 21.3 Hz, 1H), 1.18 (s, 3H), 1.13 (t, *J* = 7.1 Hz, 6H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.73 (d, *J* = 7.0 Hz, 3H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 216.5, 165.1, 148.5, 138.6, 130.3, 129.7, 129.0, 128.9, 126.4, 123.4, 120.8, 117.6, 112.2, 74.5, 71.0, 58.0, 53.4, 51.6, 45.4, 44.7, 44.0, 41.9, 40.9, 36.6, 36.1, 34.4, 30.4, 26.8, 26.4, 24.8, 16.8, 14.7, 12.6, 11.5; HRMS (ESI) m/z calculated for $C_{41}H_{55}N_4O_4$ ($M+H^+$) 667.4223 found 667.4185; HPLC purity at 254 nm: 95.9%

22-[4-(4-(*N*-Phthalimidomethyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (29) General Procedure 1 was applied with compound **9** (60 mg, 0.149 mmol), the alkyne **88** (38.9 mg, 0.149 mmol), sodium ascorbate (5.9 mg, 0.030 mmol) and $CuSO_4 \cdot 5H_2O$ (3.7 mg, 0.015 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.25 mL). Flash Chromatography (30–60% EtOAc:PE). Yield: 98 mg (99%, 0.147 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (dd, *J* = 5.5, 3.0 Hz, 2H), 7.83 (s, 1H), 7.80–7.77 (m, 2H), 7.74–7.69 (m, 2H), 7.53–7.47 (m, 2H), 6.41 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.82 (d, *J* = 8.5 Hz, 1H), 5.33 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.21 (dd, *J* = 17.3, 1.5 Hz, 1H), 5.17–5.03 (m, 2H), 4.88 (s, 2H), 3.35 (dd, *J* = 9.9, 6.5 Hz, 1H), 2.34–2.05 (m, 7H), 1.76 (m, 1H), 1.71–1.59 (m, 3H), 1.57–1.37 (m, 4H), 1.34 (s, 3H), 1.17 (s, 3H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.71 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.4, 168.0, 165.0, 147.9, 138.6, 136.5, 134.0, 132.2, 130.0, 129.2, 126.2, 123.4, 120.9, 117.6, 74.6, 71.1, 58.0, 51.7, 45.4, 44.8, 44.0, 41.9, 41.4, 36.6, 36.1, 34.4, 30.4, 26.8, 26.4, 24.8, 16.8, 14.6, 11.4; HRMS (ESI): m/z calculated for $C_{39}H_{45}N_4O_6$ ($M+H^+$) 665.3334 found 665.3301; HPLC purity at 254 nm: 99.3%

22-[4-(2-((Thymine-1-yl)methyl)phenyl)-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (30) A slightly altered General Procedure 1 was applied with the alkyne **68** (45.2 mg, 0.188 mmol) as limiting reagent, compound **9** (98 mg, 0.24 mmol), sodium ascorbate (4.5 mg, 0.023 mmol) and $CuSO_4 \cdot 5H_2O$ (5.7 mg, 0.023 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.6 mL). Flash Chromatography (MeOH:DCM, 10%). Yield: 134 mg (86%, 0.162 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.90 (s, 1H), 7.86 (s, 1H), 7.50–7.43 (m, 1H), 7.44–7.34 (m, 3H), 7.30 (d, *J* = 1.3 Hz, 1H), 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.5 Hz, 1H), 5.33 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.25–5.09 (m, 5H), 3.36 (dd, *J* = 10.5, 6.4 Hz, 1H), 2.32–2.05 (m, 6H), 1.85 (d, *J* = 1.2 Hz, 3H), 1.76 (dd, *J* = 14.6, 3.0 Hz, 1H), 1.73–1.58 (m, 3H), 1.57–1.29 (m, 8H), 1.18 (s, 3H), 1.13 (td, *J* = 14.1, 4.4 Hz, 1H), 0.88 (d, *J* = 7.0 Hz, 3H), 0.74 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 216.6, 165.1, 164.0, 151.4, 147.5, 140.8, 138.8, 134.2, 130.1, 129.7, 129.5, 129.4, 128.6, 123.7, 117.8, 110.9, 74.7, 71.4, 58.1, 51.8, 48.6, 45.6, 44.9, 44.2, 42.1, 36.7, 36.2, 34.5, 30.5, 27.0, 26.6, 25.0, 17.0, 14.8, 12.4, 11.6; HRMS (ESI): m/z calculated for $C_{36}H_{45}N_5O_6$($M+H^+$) 644.3443 found 644.3413; HPLC purity at 254 nm: 99.7%

22-[4-(3-((Thymine-1-yl)methyl)phenyl)-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (31) General Procedure 1 was applied with compound **9** (81 mg, 0.201 mmol), the alkyne **69** (48 mg, 0.201 mmol), sodium ascorbate (2.5 mg, 0.020 mmol) and $CuSO_4 \cdot 5H_2O$ (5.0 mg, 0.020 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.5 mL). Flash Chromatography (MeOH:CH_2Cl_2, 0% → 1% → 2%). Yield: 102 mg (79%, 0.158 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.33 (br s, 1H), 7.95 (s, 1H), 7.82 (d, *J* = 1.7 Hz, 1H), 7.79 (d, *J* = 7.8 Hz, 1H), 7.44 (td, *J* = 7.7, 1.4 Hz, 1H), 7.31–7.27 (m, 1H), 7.05 (d, *J* = 1.4 Hz, 1H), 6.41 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.82 (d, *J* = 8.5 Hz, 1H), 5.32 (dd, *J* = 11.1, 1.7 Hz, 1H), 5.24–5.06 (m, 3H), 4.93 (s, 2H), 3.37 (dd, *J* = 10.5, 6.4 Hz, 1H), 2.36–2.04 (m, 6H), 1.88 (d, *J* = 1.3 Hz, 3H), 1.80–1.38 (m, 8H), 1.37–1.31 (m, 4H), 1.20–1.07 (m, 4H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.72 (d, *J* = 6.9 Hz, 3H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 216.6, 165.0, 164.0, 151.3, 147.6, 139.7, 138.6, 136.3, 131.2, 129.7, 127.9, 125.9, 125.6, 121.4, 117.5, 111.4, 74.5, 71.2, 58.0, 51.7, 51.0, 45.4, 44.7, 44.0, 41.9, 36.6, 36.1, 34.4, 30.3, 26.8, 26.4, 24.8, 16.8, 14.6, 12.4, 11.4; HRMS (ESI) m/z calculated for $C_{36}H_{46}N_5O_6$ ($M+H^+$) 644.3448 found 644.3351; HPLC purity for 254 nm: 96.0%

22-[4-(4-((Thymine-1-yl)methyl)phenyl)-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (32) General Procedure 1 was applied with compound **9** (50 mg, 0.124 mmol), the alkyne **70** (31 mg, 0.124 mmol), sodium ascorbate (2.2 mg, 0.013 mmol) and $CuSO_4 \cdot 5H_2O$ (3.3 mg, 0.013 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.5 mL). Flash Chromatography (MeOH:CH_2Cl_2, 0% → 1% → 2% → 5%). Yield: 82 mg (97%, 0.127 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.80 (s, 1H), 7.88 (s, 1H), 7.85 (d, *J* = 8.3 Hz, 2H), 7.37 (d, *J* = 8.3 Hz, 2H), 7.01 (d, *J* = 1.3 Hz, 1H), 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.5 Hz, 1H), 5.34 (dd, *J* = 10.9, 1.5 Hz, 1H), 5.25–5.05 (m, 3H), 4.92 (s, 2H), 3.36 (dd, *J* = 10.5, 6.5 Hz, 1H), 2.33–2.05 (m, 5H), 1.90 (d, *J* = 1.2 Hz, 3H), 1.81–1.59 (m, 4H), 1.54–1.38 (m, 4H), 1.35 (s, 3H), 1.18 (s, 3H), 1.16–1.08 (m, 1H), 0.88 (d, *J* = 7.0 Hz, 3H), 0.72 (d, *J* = 7.1 Hz, 3H).; ^{13}C-NMR (101 MHz, $CDCl_3$) δ 11.4, 12.4, 14.6, 16.8, 24.8, 26.4, 26.8, 30.3, 34.4, 36.1, 36.6, 41.9, 44.0, 44.8, 45.4, 50.8, 51.7, 57.9, 71.1, 74.5, 111.4, 117.5, 121.1, 126.5, 128.5, 130.6, 138.5, 139.6, 147.6, 151.0, 163.8, 165.0, 216.5; HRMS (ESI) m/z calculated for $C_{36}H_{46}N_5O_6$ ($M+H^+$) 644.3448 found 644.3321; HPLC purity at 254 nm: 97.5%

22-[4-(2-((Adenine-9-yl)methyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (33) General Procedure 1 was applied with compound **9** (107 mg, 0.265 mmol), the alkyne **71** (66 mg, 0.265 mmol), sodium ascorbate (5.9 mg, 0.030 mmol) and $CuSO_4 \cdot 5H_2O$ (6.5 mg, 0.026 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.25 mL). Flash Chromatography (MeOH:DCM, 5–10%). Yield: 111 mg (64%, 0.170 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.38 (s, 1H), 7.99 (s, 1H), 7.86 (s, 1H), 7.57–7.48 (m, 1H), 7.42–7.30 (m, 3H), 6.43 (dd, *J* = 17.3, 11.0 Hz, 1H), 5.85 (d, *J* = 8.5 Hz, 1H), 5.75 (d, *J* = 7.6 Hz, 4H), 5.35 (dd, *J* = 11.0, 1.4 Hz, 1H), 5.26–5.07 (m, 3H), 3.37 (s, 1H), 2.36–2.06 (m, 5H), 1.81–1.31 (m, 11H), 1.22–1.08 (m, 4H), 0.89 (d, *J* = 7.0 Hz, 3H), 0.74 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.5, 164.9, 155.5, 153.1, 147.4, 138.7, 134.0, 130.0, 129.7, 129.2, 129.0, 128.6, 123.4, 117.6, 74.6, 71.3, 58.0, 51.7, 45.4, 45.3, 44.8, 44.1, 41.9, 36.6, 36.1, 34.4, 30.4, 26.8, 26.5, 24.8, 16.8, 14.7, 11.5; HRMS (ESI): m/z calculated for $C_{36}H_{44}N_8O_4$ ($M+H^+$) 653.3558 found 653.3596; HPLC purity at 254 nm: 99.1%

22-[4-(3-((Adenine-9-yl)methyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (34) A slightly altered General Procedure 1 was applied with the alkyne **72** (34.1 mg, 0.137 mmol) as limiting reagent, compound **9** (60.8 mg, 0.15 mmol), sodium ascorbate (4.6 mg, 0.023 mmol) and $CuSO_4 \cdot 5H_2O$ (5.6 mg, 0.022 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.5 mL). Flash

Chromatography (MeOH:DCM, 10%). Yield: 70.7 mg (79%, 0.108 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.39 (s, 1H), 7.88–7.79 (m, 3H), 7.76 (dt, *J* = 7.8, 1.4 Hz, 1H), 7.41 (t, *J* = 7.7 Hz, 1H), 7.28–7.23 (m, 1H), 6.40 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.96–5.77 (m, 3H), 5.41 (s, 2H), 5.32 (dd, *J* = 11.0, 1.4 Hz, 1H), 5.23–5.02 (m, 3H), 3.36 (s, 1H), 2.30–1.95 (m, 7H), 1.79–1.58 (m, 4H), 1.44 (m, 2H), 1.34 (s, 4H), 1.17 (s, 3H), 1.15–1.07 (m, 1H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.71 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 216.6, 165.1, 155.8, 153.4, 147.6, 140.4, 138.8, 136.5, 131.3, 129.8, 127.8, 126.0, 125.3, 121.3, 117.5, 74.7, 71.3, 58.1, 51.8, 47.2, 45.5, 44.9, 44.2, 42.0, 36.7, 36.2, 34.5, 31.0, 30.4, 26.9, 26.7, 24.9, 16.9, 14.7, 11.6, 1.1; HRMS (ESI): m/z calculated for $C_{36}H_{44}N_8O_4$ ($M+H^+$) 653.3558 found 653.3559; HPLC purity at 254 nm: 99.1%

22-[4-(4-((Adenine-9-yl)methyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (35) General Procedure 1 was applied with compound **9** (65 mg, 0.161 mmol), the alkyne **73** (40 mg, 0.161 mmol), sodium ascorbate (6.4 mg, 0.032 mmol) and $CuSO_4{\cdot}5H_2O$ (4.0 mg, 0.016 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.25 mL). Flash Chromatography (MeOH:DCM, 2–10%). Yield: 84 mg (80%, 0.129 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (s, 1H), 7.86 (s, 1H), 7.82 (d, *J* = 7.8 Hz, 2H), 7.35 (d, *J* = 7.8 Hz, 2H), 6.41 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.82 (d, *J* = 8.5 Hz, 3H), 5.40 (br s, 2H), 5.33 (d, *J* = 11.1 Hz, 1H), 5.24–5.05 (m, 3H), 3.49 (s, 2H), 3.36 (d, *J* = 6.3 Hz, 1H), 2.23 (m, 4H), 2.09 (m, 2H), 1.84–1.37 (m, 9H), 1.34 (s, 4H), 1.32–1.24 (m, 1H), 1.17 (s, 3H), 1.15–1.07 (m, 1H), 0.87 (d, *J* = 6.9 Hz, 3H), 0.72 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 216.5, 165.0, 147.6, 138.6, 135.5, 130.6, 128.4, 126.5, 121.1, 117.6, 77.2, 74.6, 71.2, 58.0, 51.7, 45.4, 44.8, 44.1, 41.9, 36.6, 36.1, 34.4, 30.4, 26.8, 26.4, 24.8, 16.8, 14.6, 11.5; HRMS (ESI): m/z calculated for $C_{36}H_{45}N_8O_4$ ($M+H^+$) 653.3558 found 653.3538; HPLC purity at 254 nm: 96.3%

22-[4-(2-((6-(*N*,*N*-Diethylamino)-9H-purin-9-yl)methyl)phenyl)-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (36) A slightly altered General Procedure 1 was applied with the alkyne **79** (57.6 mg, 0.188 mmol) as limiting reagent, compound **9** (93.4 mg, 0.231 mmol), sodium ascorbate (5.0 mg, 0.025 mmol) and $CuSO_4{\cdot}5H_2O$ (5.5 mg, 0.022 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.5 mL). Flash Chromatography (MeOH:DCM, 0–4%). Yield: 95 mg (54%, 0.102 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.33 (s, 1H), 7.86 (s, 1H), 7.83 (s, 1H), 7.56–7.50 (m, 1H), 7.35 (td, *J* = 7.5, 1.6 Hz, 1H), 7.30 (td, *J* = 7.5, 1.6 Hz, 1H), 7.23–7.19 (m, 1H), 6.42 (dd, *J* = 17.4, 11.0 Hz,

1H), 5.83 (d, *J* = 8.5 Hz, 1H), 5.68 (s, 2H), 5.34 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.24–5.06 (m, 3H), 3.98 (s, 4H), 3.35 (dd, *J* = 10.5, 6.5 Hz, 1H), 2.31–2.19 (m, 3H), 2.18–2.10 (m, 1H), 2.09–2.07 (m, 1H), 1.84–1.38 (m, 8H), 1.37 (s, 3H), 1.28 (t, *J* = 7.0 Hz, 6H), 1.17 (s, 3H), 1.12 (td, *J* = 14.1, 4.5 Hz, 1H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.73 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.4, 164.9, 153.9, 152.6, 150.8, 147.3, 138.8, 138.6, 134.3, 129.7, 129.4, 129.1, 128.9, 128.3, 123.4, 119.5, 117.6, 74.5, 71.1, 58.0, 51.6, 45.4, 45.0, 44.8, 44.0, 43.0, 41.9, 36.5, 36.1, 34.4, 30.3, 26.8, 26.4, 24.8, 16.8, 14.7, 13.5, 11.5; HRMS (ESI): m/z calculated for $C_{40}H_{52}N_8O_4$ ($M+H^+$) 709.4184 found 709.4215; HPLC purity at 254 nm: 99.6%

22-[4-(3-((6-(*N*,*N*-Diethylamino)-9H-purin-9-yl)methyl)phenyl)-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (37) A slightly altered General Procedure 1 was applied with the alkyne **80** (58.7 mg, 0.192 mmol) as limiting reagent, compound **9** (94.7 mg, 0.23 mmol), sodium ascorbate (5 mg, 0.025 mmol) and $CuSO_4{\cdot}5H_2O$ (5.3 mg, 0.021 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.5 mL). Flash Chromatography (MeOH:DCM, 10%). Yield: 132 mg (97%, 0.186 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.38 (s, 1H), 7.85 (s, 1H), 7.83 (d, *J* = 1.8 Hz, 1H), 7.77 (dt, *J* = 7.8, 1.4 Hz, 1H), 7.74 (s, 1H), 7.40 (t, *J* = 7.7 Hz, 1H), 7.27–7.23 (m, 1H), 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.5 Hz, 1H), 5.40 (s, 2H), 5.34 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.22 (dd, *J* = 17.4, 1.5 Hz, 1H), 5.18–5.03 (m, 2H), 4.00 (s, 4H), 3.36 (dd, *J* = 10.6, 6.5 Hz, 1H), 2.33–2.07 (m, 5H), 1.77 (dd, *J* = 14.4, 3.1 Hz, 1H), 1.72–1.59 (m, 5H), 1.57–1.36 (m, 6H), 1.30 (t, *J* = 7.0 Hz, 6H), 1.18 (s, 3H), 1.18–1.07 (m, 1H), 0.88 (d, *J* = 7.0 Hz, 3H), 0.73 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 216.5, 165.2, 154.1, 152.9, 150.9, 147.9, 138.8, 138.2, 137.0, 131.2, 129.7, 127.8, 125.8, 125.4, 121.3, 119.7, 117.7, 74.7, 71.3, 58.1, 51.8, 47.0, 45.6, 44.9, 44.2, 42.0, 36.7, 36.2, 34.5, 31.0, 30.5, 26.9, 26.5, 25.0, 17.0, 14.8, 13.7, 11.6; HRMS (ESI): m/z calculated for $C_{40}H_{52}N_8O_4$ ($M+H^+$) 709.4184 found 709.4205; HPLC purity at 254 nm: 100%

22-[4-(4-((6-(*N*,*N*-Diethylamino)-9*H*-purin-9-yl)methyl)phenyl)-1*H*-1,2,3-triazol-1-y]-22-deoxypleuromutilin (38) General Procedure 1 was applied with compound **9** (60 mg, 0.149 mmol), the alkyne **81** (45.5 mg, 0.149 mmol), sodium ascorbate (5.9 mg, 0.030 mmol) and $CuSO_4{\cdot}5H_2O$ (3.7 mg, 0.015 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.00 mL). Flash Chromatography (MeOH:DCM, 0–3%). Yield: 98 mg (93%, 0.138 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.38 (s, 1H), 7.84 (s, 1H), 7.81 (d, *J* = 8.3 Hz, 2H), 7.73 (s, 1H), 7.35 (d, *J* = 8.4 Hz, 2H),

6.41 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.5 Hz, 1H), 5.39 (s, 2H), 5.34 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.21 (dd, *J* = 17.4, 1.5 Hz, 1H), 5.18–5.04 (m, 2H), 4.00 (s, 4H), 3.35 (dd, *J* = 10.6, 6.5 Hz, 1H), 2.33–2.04 (m, 5H), 1.76 (dd, *J* = 14.5, 3.1 Hz, 1H), 1.71–1.66 (m, 1H), 1.63–1.59 (m, 1H), 1.57–1.38 (m, 4H), 1.35 (s, 4H), 1.30 (t, *J* = 7.0 Hz, 6H), 1.18 (s, 3H), 1.16–1.08 (m, 1H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.72 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 216.5, 165.0, 153.9, 152.9, 150.7, 147.7, 138.6, 138.0, 136.1, 130.3, 128.3, 126.4, 121.0, 119.6, 117.6, 74.6, 71.1, 58.0, 51.7, 46.7, 45.4, 44.8, 44.0, 43.1, 41.9, 36.6, 36.1, 34.4, 30.4, 26.8, 26.4, 24.8, 16.8, 14.6, 13.5, 11.5; HRMS (ESI): m/z calculated for $C_{40}H_{53}N_8O_4$ ($M+H^+$) 709.4184 found 709.4151; HPLC purity at 254 nm: > 99.9%

22-[4-(2-((2-Amino-6-(*N*,*N*-diethylamino)-9H-purin-9-yl)methyl)phenyl)-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (39) A slightly altered General Procedure 1 was applied with the alkyne **82** (56.6 mg, 0.188 mmol) as limiting reagent, compound **9** (96 mg, 0.238 mmol), sodium ascorbate (4.5 mg, 0.023 mmol) and $CuSO_4{\cdot}5H_2O$ (5.7 mg, 0.023 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.5 mL). Flash Chromatography (MeOH:DCM, 0 - 3%). Yield: 108 mg (80%, 0.150 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (s, 1H), 7.57 (dd, *J* = 7.5, 1.6 Hz, 1H), 7.48 (s, 1H), 7.33 (m, 2H), 7.15 (dd, *J* = 7.6, 1.5 Hz, 1H), 6.42 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.5 Hz, 1H), 5.50 (s, 2H), 5.35 (dd, *J* = 11.0, 1.4 Hz, 1H), 5.26 – 5.05 (m, 3H), 4.61 (s, 2H), 3.92 (s, 4H), 3.35 (s, 1H), 2.33 – 2.05 (m, 6H), 1.76 (m, 1H), 1.72 – 1.37 (m, 6H), 1.37 (s, 5H), 1.25 (t, *J* = 7.0 Hz, 7H), 1.17 (s, 3H), 1.13 (dt, *J* = 13.8, 7.0 Hz, 1H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.72 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.6, 165.1, 159.5, 154.4, 152.9, 147.2, 138.8, 136.6, 134.7, 129.8, 129.2, 129.1, 128.3, 123.6, 117.8, 114.6, 74.7, 71.2, 58.1, 51.8, 45.5, 44.9, 44.8, 44.2, 42.7, 42.0, 36.7, 36.2, 34.5, 30.5, 26.9, 26.5, 25.0, 17.0, 14.8, 13.7, 11.6; HRMS (ESI): m/z calculated for $C_{40}H_{53}N_9O_4$ ($M+H^+$) 724.4293 found 724.4272; HPLC purity at 254 nm: 100%

22-[4-(3-((2-Amino-6-(*N*,*N*-diethylamino)-9H-purin-9-yl)methyl)phenyl)-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (40) A slightly altered General Procedure 1 was applied with the alkyne **83** (60.7 mg, 0.189 mmol) as limiting reagent, compound **9** (98.1 mg, 0.24 mmol), sodium ascorbate (5.2 mg, 0.026 mmol) and $CuSO_4{\cdot}5H_2O$ (5.6 mg, 0.022 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.5 mL). Flash Chromatography (MeOH:DCM, 10%). Yield: 132 mg (96%, 0.181 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (s, 1H), 7.79 (d, *J* = 1.8 Hz, 1H), 7.75 (dt, *J* = 7.7, 1.4 Hz, 1H),

7.46 (s, 1H), 7.38 (t, *J* = 7.7 Hz, 1H), 7.21 (dt, *J* = 7.9, 1.3 Hz, 1H), 6.41 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.5 Hz, 1H), 5.34 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.25 (s, 2H), 5.21 (dd, *J* = 17.4, 1.5 Hz, 1H), 5.18–5.03 (m, 2H), 4.59 (s, 2H), 3.92 (s, 4H), 3.35 (dd, *J* = 10.7, 6.5 Hz, 1H), 2.31–2.06 (m, 5H), 1.76 (dd, *J* = 14.5, 3.1 Hz, 1H), 1.72–1.59 (m, 6H), 1.58–1.39 (m, 3H), 1.36 (s, 4H), 1.25 (t, *J* = 7.0 Hz, 6H), 1.18 (s, 3H), 1.18–1.06 (m, 1H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.73 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 216.6, 165.2, 159.7, 154.4, 153.0, 148.0, 138.8, 137.4, 136.0, 131.1, 129.6, 127.8, 125.6, 125.3, 121.2, 117.8, 114.7, 74.7, 71.3, 58.2, 51.8, 46.5, 45.6, 44.9, 44.2, 42.7, 42.0, 36.7, 36.2, 34.5, 30.5, 27.0, 26.5, 25.0, 17.0, 14.8, 13.7, 11.6; HRMS (ESI): m/z calculated for $C_{40}H_{53}N_8O_4$ ($M+H^+$) 724.4293 found 724.4311; HPLC purity at 254 nm: 100%

22-[4-(4-((6-(4-Methylpiperazin-1-yl)-9*H*-purin-9-yl)methyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (41) General Procedure 1 was applied with compound **9** (81 mg, 0.20 mmol), the alkyne **84** (68 mg, 0.20 mmol), sodium ascorbate (2.5 mg, 0.02 mmol) and $CuSO_4{\cdot}5H_2O$ (5.0 mg, 0.02 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.5 mL). Flash Chromatography (MeOH:CH_2Cl_2, 0% → 1% → 2% → 5%). Yield: 70.0 mg (48%, 0.095 mmol); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.39 (s, 1H), 7.85 (s, 1H), 7.83 (m, 1H), 7.76 (s, 2H), 7.40 (t, *J* = 7.7 Hz, 1H), 7.24 (dt, *J* = 7.7, 1.4 Hz, 1H), 6.41 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.5 Hz, 1H), 5.41 (s, 2H), 5.33 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.24 – 5.04 (m, 3H), 4.35 (s, 4H), 3.36 (d, *J* = 6.4 Hz, 1H), 2.57 (t, *J* = 5.1 Hz, 4H), 2.36 (s, 3H), 2.32 – 2.05 (m, 6H), 1.81 – 1.58 (m, 4H), 1.57 – 1.39 (m, 3H), 1.36 (d, *J* = 2.9 Hz, 3H), 1.33 – 1.24 (m, 1H), 1.20 – 1.08 (m, 4H), 0.88 (d, *J* = 7.0 Hz, 3H), 0.73 (d, *J* = 7.1 Hz, 3H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 216.5, 165.0, 152.6, 151.1, 147.6, 138.9, 138.6, 138.2, 138.0, 136.6, 131.1, 129.6, 127.6, 125.7, 125.1, 121.2, 117.6, 74.5, 71.1, 57.9, 55.1, 53.4, 51.6, 46.1, 45.4, 44.7, 44.0, 41.9, 36.5, 36.1, 34.4, 30.3, 26.8, 26.4, 24.8, 16.8, 14.6, 11.5; HRMS (ESI) m/z calculated for $C_{42}H_{56}N_8O_4$ ($M+H^+$) 736.4425 found 736.4295; HPLC purity at 254 nm: 99.2%

22-[4-(4-((6-(4-Methylpiperazin-1-yl)-9*H*-purin-9-yl)methyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (42) General Procedure 1 was applied with compound **9** (55 mg, 0.136 mmol), the alkyne **85** (45.3 mg, 0.136 mmol), sodium ascorbate (5.4 mg, 0.027 mmol) and $CuSO_4{\cdot}5H_2O$ (3.4 mg, 0.014 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.25 mL). Flash Chromatography (MeOH:DCM, 2–10%). Yield: 88 mg (88%, 0.120 mmol); ^{1}H NMR (400 MHz,

$CDCl_3$) δ 8.39 (s, 1H), 7.84 (s, 1H), 7.81 (d, *J* = 8.3 Hz, 2H), 7.74 (s, 1H), 7.34 (d, *J* = 8.4 Hz, 2H), 6.41 (dd, *J* = 17.4, 11.0 Hz, 1H), 5.83 (d, *J* = 8.5 Hz, 1H), 5.40 (s, 2H), 5.34 (dd, *J* = 11.0, 1.5 Hz, 1H), 5.21 (dd, *J* = 17.4, 1.5 Hz, 1H), 5.18–5.03 (m, 2H), 4.35 (s, 4H), 3.35 (dd, *J* = 10.0, 6.5 Hz, 1H), 2.56 (t, *J* = 5.1 Hz, 4H), 2.35 (s, 3H), 2.32–2.04 (m, 5H), 1.82–1.72 (m, 2H), 1.65 (m, 2H), 1.52 (s, 1H), 1.49–1.38 (m, 3H), 1.37–1.24 (m, 5H), 1.18 (s, 3H), 1.16–1.07 (m, 1H), 0.87 (d, *J* = 7.0 Hz, 3H), 0.72 (d, *J* = 7.0 Hz, 3H) (App. 17.A); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.5, 165.0, 154.0, 152.7, 151.1, 147.7, 138.6, 138.2, 135.9, 130.4, 128.2, 126.4, 121.0, 119.9, 117.6, 74.5, 71.1, 58.0, 55.2, 51.7, 46.8, 46.2, 45.4, 44.8, 44.0, 41.9, 36.6, 36.1, 34.4, 30.4, 26.8, 26.4, 24.8, 16.8, 14.6, 11.5; HRMS (ESI): m/z calculated for $C_{41}H_{54}N_9O_4$ ($M+H^+$) 736.4293 found 736.4297; HPLC purity at 254 nm: 98.3%

22-[4-(4-((6-(*N*-(2-Aminoethyl)amino)-9*H*-purin-9-yl)methyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (43) General Procedure 1 was applied with compound **9** (60 mg, 0.149 mmol), the alkyne **86** (43.3 mg, 0.149 mmol), sodium ascorbate (5.9 mg, 0.030 mmol) and $CuSO_4{\cdot}5H_2O$ (3.7 mg, 0.015 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.25 mL). Flash Chromatography (NH_3:MeOH:DCM, 0:5:95 → 0:20:80 → ½: 19.5:80). Yield: 57 mg (55%, 0.082 mmol); ^{1}H NMR (400 MHz, DMSO) δ 8.50 (s, 1H), 8.33 (s, 1H), 8.25 (s, 1H), 7.82 (d, *J* = 8.2 Hz, 2H), 7.40 (d, *J* = 8.3 Hz, 2H), 6.12 (dd, *J* = 17.8, 11.2 Hz, 1H), 5.57 (d, *J* = 8.3 Hz, 1H), 5.45 – 5.28 (m, 4H), 5.12 (dd, *J* = 17.8, 1.8 Hz, 1H), 5.04 (dd, *J* = 11.2, 1.8 Hz, 1H), 4.56 (s, 1H), 3.62 (s, 3H), 2.90 (d, *J* = 8.3 Hz, 2H), 2.40 (s, 1H), 2.24 – 2.00 (m, 4H), 1.61 (q, *J* = 12.2, 10.8 Hz, 2H), 1.54 – 1.41 (m, 1H), 1.36 (d, *J* = 15.9 Hz, 2H), 1.25 (d, *J* = 12.9 Hz, 2H), 1.22 (s, 3H), 1.07 (s, 3H), 0.99 (td, *J* = 13.8, 4.4 Hz, 1H), 0.80 (d, *J* = 6.9 Hz, 3H), 0.63 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ ^{13}C NMR (101 MHz, DMSO) δ 216.9, 165.4, 152.4, 145.8, 140.8, 140.7, 136.6, 130.0, 128.1, 125.4, 122.8, 115.3, 72.4, 70.6, 57.0, 51.2, 45.9, 44.8, 44.0, 43.3, 41.4, 36.3, 36.1, 33.9, 29.9, 28.4, 26.4, 24.3, 16.0, 14.1, 11.4; HRMS (ESI): m/z calculated for $C_{38}H_{50}N_9O_4$ ($M+H^+$) 696.3980 found 696.3988; HPLC purity at 254 nm: 96.7%

22-[4-(4-((6-Guanidino-9*H*-purin-9-yl)methyl)phenyl)-1*H*-1,2,3-triazol-1-yl]-22-deoxypleuromutilin (44) General Procedure 1 was applied with compound **9** (60 mg, 0.149 mmol), the alkyne **87** (43.5 mg, 0.149 mmol), sodium ascorbate (5.9 mg, 0.030 mmol) and $CuSO_4{\cdot}5H_2O$ (3.7 mg, 0.015 mmol) in degassed t-BuOH:H_2O (1:1 v/v, 2.25 mL). Flash

Chromatography (MeOH:DCM, 2–20%). Yield: 55 mg (53%, 0.079 mmol); ^{1}H NMR (400 MHz, DMSO) δ 8.67 (s, 1H), 8.60 (s, 1H), 8.51 (s, 1H), 8.39 (s, 3H), 7.83 (d, *J* = 8.3 Hz, 2H), 7.43 (d, *J* = 8.1 Hz, 2H), 6.12 (dd, *J* = 17.8, 11.2 Hz, 1H), 5.57 (d, *J* = 8.3 Hz, 1H), 5.53 (s, 2H), 5.36 (q, *J* = 17.6 Hz, 2H), 5.11 (dd, *J* = 17.8, 1.8 Hz, 1H), 5.04 (dd, *J* = 11.2, 1.7 Hz, 1H), 4.55 (d, *J* = 6.0 Hz, 1H), 3.41 (t, *J* = 6.1 Hz, 1H), 2.43–2.36 (m, 1H), 2.23–1.97 (m, 4H), 1.62 (t, *J* = 12.5 Hz, 2H), 1.53–1.41 (m, 1H), 1.36 (d, *J* = 16.0 Hz, 2H), 1.31–1.23 (m, 2H), 1.21 (s, 3H), 1.07 (s, 3H), 0.99 (td, *J* = 13.8, 4.4 Hz, 1H), 0.80 (d, *J* = 6.9 Hz, 3H), 0.62 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 216.9, 165.4, 156.4, 150.7, 145.8, 140.7, 136.0, 130.2, 128.2, 125.5, 122.8, 115.3, 72.4, 70.6, 57.0, 51.2, 48.5, 46.3, 44.8, 44.0, 43.3, 41.4, 36.3, 36.1, 33.9, 30.9, 29.9, 28.4, 26.4, 24.3, 16.0, 14.1, 11.4; HRMS (ESI): m/z calculated for $C_{37}H_{47}N_{10}O_4$ ($M+H^+$) 695.3776 found 695.3747, HPLC purity at 254 nm: 95.4%

In Vitro susceptibility assay (MIC) and minimum bactericidal concentration (MBC).

MRSA USA300 FPR3757[30] was used for susceptibility evaluation of all test compound in combination with Mueller-Hinton (MH) broth. The clinical isolates MRSA (55508, 52518, 4828) and MRSE 933010 were obtained via Odense University Hospital (OUH) and Statens Seruminstitut (SSI). Testing was done using a 96-well plate, micro-broth serial dilution assay and was conducted in accordance with the guidelines presented by EUCAST[41] (inoculum = $5.0 \cdot 10^5$ CFU mL^{-1}). One compound was tested per plate, using three single colony overnight-cultures for inoculation, and constituted one singular MIC replicate. Each culture was diluted to a concentration of OD_{600} = 0.1 before they were diluted an additional 100x and added to each well (100 µL) already containing the serial diluted antibacterial compound in MH (100 µL) with the final concentration ranging between 0.015–16 µg mL^{-1}. Plates were covered in aluminium foil and incubated at 37 ºC for 20–24 h. MIC was established as the concentration at which no bacterial growth was visible. Inocula was verified by consecutive 10x dilution of the OD_{600} = 0.001 bacterial solution in 1x phosphate-buffered saline followed by plating onto MH/agar plates.

Assessment of minimal bactericidal concentration was conducted on a completed MIC replicate by plating 10 µL aliquots from the wells downstream of the MIC-value, i.e. at 2x MIC, 4x MIC, 8x MIC and 16x MIC. The plates were incubated for 20 h. after which the viable colonies were counted. A bactericidal result is defined as the concentration of antibacterial agent at which a 99.9% reduction of the original standard inoculum is observed.

MM-GBSA molecular model.

The molecular model setup and evaluation was performed using the Maestro program from the Schrödinger suite (version 2019-1) in combination with the X-ray crystal structure of the large ribosomal subunit of *Staphylococcus aureus* (NTCT 8325) in complex with lefamulin (**5**) (pdb: 5HL7).[17] Initial preprocessing of the full ribosomal subunit was computationally infeasible. To reduce the size of the system, a spherical cut of 30 Å was made (with full residues) around the native ligand, thus fully encompassing the PTC. To verify the reliability of the cut-out PTC model, lefamulin **5** was redocked with extra precision (XP) Glide (1.0 van der Walls atom scaling), with the result coinciding with the crystalized pose. Glide XP poses were computed for **7** and **35** and used as minimized starting points for the Prime MM-GBSA model, which is able to grant an approximation of (solvated) binding mode using the VSGB 2.1 implicit solvent model. An induced-fit was not included in the MM-GBSA calculations (no flexibility of proximal residues/nucleotides). Pose visualization was done using VMD 1.9.3.

Cell viability assays.

The viability of the cells was investigated using the CellTiter-Glo® assay measuring cellular ATP levels. Initially, compounds, except SDS, were dissolved in DMSO. Then, compounds were diluted in HBSS buffer (buffered with 10 mM HEPES adjusted to pH 7.4 with NaOH) to a final DMSO concentration < 1% for the highest concentrations of **35**, valnemulin (**3**) and retapamulin (**4**). During the experiments, plates were protected from light with aluminium foil. The following concentrations were investigated 0.011, 0.1, 1.09, 10.9, 35.8, and 108.6 μg mL^{-1} for **35**; and 0.01, 0.10, 10.0, 33.0, and 100 μg mL^{-1} for valnemulin (**3**) as well as 0.1 and 33.0 μg mL^{-1} for retapamulin (**4**). These concentrations were selected to cover a broad concentration range around the IC_{50} value (14.9 μg mL^{-1}) reported for cytotoxicity of valnemulin (**3**) in African green monkey kidney epithelial cells (Vero) measured by the MTT assay.[38] Data in Figure 6 shows the effect on cell viability of the highest investigated compound concentration.

For the CellTiter-Glo® assay, the growth medium was aspirated. 50 μL test compound was added to the wells and incubated for 3 hours at 220 rpm and 37 °C. For measurement of ATP, the CellTiter-Glo® Reagent (CellTiter-Glo® Buffer and CellTiter-Glo® Substrate) was thawed and equilibrated to room temperature and mixed prior to use. The plate was equilibrated for 10 min at room temperature and 30 μL of CellTiter-Glo® 2.0 Reagent was added directly to the wells. The

plate was mixed on an orbital shaker for 2 min. at room temperature. The plate was then incubated at room temperature for 10 min. to stabilize the luminescent signal. The viability was assessed by normalizing the luminescence recorded to the luminescence obtained in control cells incubated in HBSS buffer only.

Cellular toxicity of the test compounds was also investigated using a Thiazolyl Blue Tetrazolium Bromide (MTT) assay. The growth medium was aspirated, and 50 µL test compound, SDS (positive control) or HBSS buffer (negative control) was added and the cells were incubated for 3 hours at 220 rpm and 37°C. Then, 30 µL 1.33 mg mL^{-1} MTT (final concentration of 0.5 mg mL^{-1}) was added and the cells were incubated for an additional 2 hours. To dissolve the formed formazan salt, 200 µL of 10 mM HCl, 50% isobutanol and 10% SDS was added directly to the well and the cells were incubated overnight at 4°C. Absorbance was measured at 590 nm.

Associated content

Experimental procedures that are not covered in the main text, for compounds **8**–**9**, **48**–**89** and **92**–**97** are listed in the Supporting Information. ^{1}H NMR and ^{13}C NMR spectra as well as HPLC profiles (254 nm) for conjugates **10**–**44** are also supplied herein alongside their Molecular Formula Strings. This material is available free of charge at http://pubs.acs.org

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Notes

A patent application related to compound **35** and derivatives has been filed by University of Southern Denmark with Christoffer V. Heidtmann and Poul Nielsen listed as inventors.

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Abbreviations

MM-GBSA molecular mechanics generalized Born surface area; PTC peptidyl transferase center; TBAF tetrabutylammonium fluoride; PSA polar surface area

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