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New antituberculotics originated from salicylanilides with promising in vitro activity against atypical mycobacterial strains

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ABSTRACT

A new series of 30 *N*-protected amino acid esters were prepared as a part of ongoing search for new antituberculosis active salicylanilides. The esters possess high in vitro activity against *Mycobacterium tuberculosis*, *Mycobacterium avium,* and two strains of *Mycobacterium kansasii*, where one is an isolate from the patient, with MIC in the range 1–32 μmol/L for all tested strains. The prepared esters can be considered as prodrugs with better bio-availability and as more efficient transport forms through the mycobacterial cell membranes due to the higher lipophilicity. The experimental and calculated lipophilicity, stability, antituberculotic activity, cytotoxicity as well as the quantitative structure–activity relationships (QSARs) explored by the Intelligent Problem Solver (IPS) in Trajan Neural Network Simulator 6.0 are presented.

1. Introduction

Tuberculosis (TB) was one of the first identified infectious diseases. Nevertheless, it has become the most deadly infectious disease on the planet killing nearly 2 million people per year. It is estimated that about one-third of the world's population (2 billion people) is infected with Mycobacterium tuberculosis (MTB) and 10% of them will progress to the active disease. The highest incidence and burden of disease is observed in India, China, Indonesia, Nigeria, and Bangladesh with the rates ranging from 293 to 102 new cases per 100,000 people of country's population per year.[1]

Excessive use of antibiotics is generally accepted to be the main reason for increased antibiotic resistance among bacteria.[2,3] Resistance to antimicrobial agents is an unavoidable side effect of their use and goes hand in hand with an inexorable drive of bacterial evolution. The situation in prevention and control of tuberculosis is further fuelled by the deadly rise of multidrug-resistant TB (MDR-TB).[4] Currently available antibiotics are ineffectual with respect to these MDR strains. Particularly in countries of Africa, tuberculosis incidence has been increasing, primarily as a result of the HIV/AIDS epidemic. This co-infection (HIV/AIDS–TB) is one of the major problems in the treatment and control of TB.[5]

Over the past 50 years, no new drug classes have been introduced to the treatment of tuberculosis.[1] Currently, patients require between 6 and 9 months of treatment. This long period leads to the lack of compliance, which in turn can be responsible for the relapse and emergence of MDR-TB strains.[6] Hence, there is an urgent need to develop new, potent, and fast acting anti-tuberculosis drugs with low toxicity profiles that can be used in conjunction with drugs used for HIV infection treatment and are active against both actively growing and latent infections.

Salicylanilides (2-hydroxy-*N*-phenylbenzamides) have been the subject of intensive interest in medicinal chemistry, due to a wide variety of their biological activities, particularly antimycobacterial and antifungal activities.[7–10] They exhibit high effect against *M. tuberculosis* and isoniazid resistant strains *Mycobacterium* avium and *Mycobacterium kansasii*.[11] Even if their mechanism of action is still under investigation, they serve as inhibitors of protein kinase epidermal growth factor receptor (EGFR PTK).[12] They are generally designed to compete with ATP for binding in catalytic

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domain of tyrosin kinase.[13,14] The latest studies specified them also as selective inhibitors of interleukin-12p40 production that plays a specific role in initiation, expansion, and control of cellular response to tuberculosis.[15,16]

2. Chemistry

Modifications of salicylanilides **1** should bring prodrugs with improved physico-chemical properties important for passing better through the cell membrane and lower toxicity. Previously studied salicylanilide acetates exhibited promising antimycobacterial activity against *M. tuberculosis* and some atypical mycobacterial strains *M. avium* and *M. kansasii*, but their toxicity did not decrease significantly in comparison with salicylanilides.[17] Therefore a new prodrug form of amino acid esters and salicylanilides was proposed. Desired compounds will have higher lipophilicity than starting salicylanilides. Several perspective models of salicylanilide amino acid esters with high antifungal activity confirm our suggestions. The respective results were published recently.[10]

On the basis of the known results, the series of the above-mentioned salicylanilide derivatives was synthesized in two steps. Anilines and salicylic acids were reacted under microwave heating, using PCl_3 in chlorobenzene, to form starting salicylanilides **1**. In the second step, *N*-benzyloxycarbonyl amino acids (*N*-Cbz-AA) **2** were esterified with salicylanilides **1** by using *N,N′*-dicyclohexylcarbodiimide activation as an optimal method.[18] Lipophilic *N*-Cbz-AA such as glycin, *R*/*S*-alanine, *R*/*S*-valine, and *R*/*S*-phenylalanine were used for esterification. As expected, in most cases the reaction gave the desired esters **3** in high yields. When Cbz-Gly and Cbz-L-Ala were esterified with salicylanilide that did not have halogen in position $C'_{(3)}$, the major isolated product was unambiguously identified by MS and gHMBC 2D NMR experiments as benzoxazepine-2,5-dione.[19] The general synthetic pathway is shown in Scheme 1.

3. Antimycobacterial activity of the prepared compounds

All the prepared *N*-Cbz-amino acid esters **3** were tested in vitro for their antimycobacterial activity in the National Reference Laboratory for *Mycobacterium kansasii*, against *M. tuberculosis* (*M. tbc.*) 331/88 and moreover for some non-tuberculous strains such as *M. avium* (330/88) and *M. kansasii* (235/80 and 6509/96). The anti-TB screening results of compounds **3** are summarized in Table 1.

4. Lipophilicity properties of the prepared compounds

Hydrophobicities (log *P*/Clog *P* values) of the studied compounds **3** were calculated using two commercially available programs (CHEMDRAW Ultra 10.0 and ACD/log P) and measured by means of RP-HPLC determination of capacity factors *k* with subsequent calculation of log *k*.[20]

Both used programs do not resolve lipophilicity parameters within the series of enantiomers. Program CHEMDRAW did not resolve various lipophilicity values of individual positional isomers, that is, the same log P/Clog *P* data were calculated for series 4-Cl (**3a**–**3x**) and series 5-Cl (**3y**–**3dd**) as well as for $C_{(3)}$/$C_{(4)}$ substitution in phenylcarbamoyl moiety, that is, compounds **3a**–**3g**, **3h**–**3l**.

Experimentally determined values of log *k* are in accordance with the calculated outcomes for both groups (series 4-Cl and series 5-Cl) of compounds **3**. Lipophilicity factor log *k* as well as calculated log *P* (ACD/log *P*) and log *P*/Clog *P* values (CHEMDRAW) increase in dependence on amino acid type (glycine, alanine, valine, and phenylalanine), that is, lipophilicity of the compounds substituted in R^3 increases: $H < CH_3 < CH(CH_3)_2 < CH_2C_6H_5$. The compounds substituted by chlorine in $C_{(5)}$ position of benzene ring showed higher lipophilicity according to program ACD/log P than the compounds with $C_{(4)}$ chlorine substitution, but experimental log *k* for $C_{(4)}$-series is higher than that for $C_{(5)}$-series. As expected, lipophilicity of the compounds substituted in the anilide part of the molecule increases: Cl < Br < diCl. Compounds **3a**–**3g** substituted by chlorine in $C'_{(3)}$ position showed higher lipophilicity according to program ACD/log P than compounds **3h**–**3l** with $C'_{(4)}$ chlorine substitution, but experimental log k for $C'_{(4)}$ chlorine substitution is higher. The results are shown in Table 1.

Log *k* data specify lipophilicity within this series of the discussed compounds and all the chiral compounds were measured several times giving the same results. The determined differences in log k parameters for individual *R*/*S*-enantiomers cannot be explained on the basis of the results presented here.

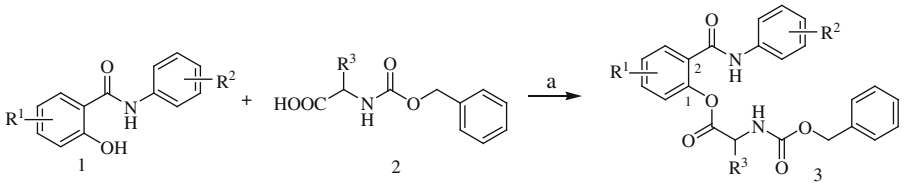
5. Cytotoxicity assay

Cytotoxicity assessment made on human intestinal cell line HCT-8 (ECACC, UK) showed medium cytotoxicity for all measured compounds, in comparison to the standard INH. The selectivity index (SI) is defined as the ratio of the measured EC_{50} (mammalian cell toxicity) to the MIC. All observed compounds have SI value higher than 10, it means the compounds may be considered suitable for further screening[21] (Table 2).

6. Study of hydrolytic stability of the most active compounds against M. avium and *M. kansasii* (3n, 3o, 3q, 3bb, 3cc, 3dd)

The studied derivatives were designed as a new prodrug form of salicylanilides with prolonged liberation. To simulate blood or serum and afflicted tissue, the stability in two types of phosphate buffer at pH 7.4 (7×10^{-2} M, 37 °C) and pH 5.5 (7×10^{-2} M, 37 °C) was measured, using UV/vis spectroscopy.[22] During the hydrolysis we observed an absorbance decrease at 224 nm. This absorption band decrease corresponds to the rate of hydrolyses. From experimental data measured at 224 nm, the half-times ($t_{1/2}$) and rate constants were calculated. The results are summarized in Table 2.

Experimental results for both types of hydrolysis are comparable, acidic hydrolysis is slightly faster. The influence of amino acid on the rate of hydrolysis requires further experiments. The hydro-



Scheme 1. Synthesis of investigated salicylanilide derivatives. Reagents and condition: (a) DCC, DMF, −10 °C.

Table 1
Evaluation of Cbz-amino acid esters 3

Compd	R^1	R^2	R^3	MIC (μM)										Lipophilicity factor		
				M. tbc 331/88		*M. avium* 330/88		*M. kansasii* 235/80			*M. kansasii* 6509/96			log *k*	log *P* ACD/log *P*	log *P*/Clog *P* ChemOffice
				14d	21d	14d	21d	7d	14d	21d	7d	14d	21d			
3a	4-Cl	3-Cl	H	4	8	16	32	16	16	16	8	16	16	0.6119	5.04 ± 0.48	4.71/5.14625
3b	4-Cl	3-Cl	(*S*)-CH_3	4	8	32	62.5	16	16	16	8	16	16	0.6995	5.39 ± 0.48	5.20/5.45525
3c	4-Cl	3-Cl	(*R*)-CH_3	8	8	32	32	16	16	16	8	16	16	0.6990	5.39 ± 0.48	5.20/5.45525
3d	4-Cl	3-Cl	(*S*)-CH–$(CH_3)_2$	4	4	16	16	8	16	16	8	16	16	0.8972	6.27 ± 0.48	6.09/6.38325
3e	4-Cl	3-Cl	(*R*)-CH–$(CH_3)_2$	8	8	32	32	8	16	16	8	16	16	0.8954	6.27 ± 0.48	6.09/6.38325
3f	4-Cl	3-Cl	(*S*)-CH_2-phenyl	16	16	32	62.5	32	32	32	32	32	32	1.0465	7.32 ± 0.49	6.88/6.87325
3g	4-Cl	3-Cl	(*R*)-CH_2-phenyl	4	4	16	32	8	16	16	8	16	16	1.0476	7.32 ± 0.49	6.88/6.87325
3h	4-Cl	4-Cl	(*R*)-CH_3	4	4	16	16	8	16	16	8	8	16	0.7294	5.35 ± 0.48	5.20/5.45525
3i	4-Cl	4-Cl	(*S*)-CH–$(CH_3)_2$	4	4	16	16	8	16	16	8	8	16	0.9417	6.23 ± 0.48	6.09/6.38325
3j	4-Cl	4-Cl	(*R*)-CH–$(CH_3)_2$	4	4	16	16	8	8	16	8	8	16	0.9421	6.23 ± 0.48	6.09/6.38325
3k	4-Cl	4-Cl	(*S*)-CH_2-phenyl	4	4	16	16	8	16	16	8	16	16	1.0971	7.03 ± 0.58	6.88/6.87325
3l	4-Cl	4-Cl	(*R*)-CH_2-phenyl	4	4	16	16	8	8	8	8	8	16	1.0678	7.28 ± 0.40	6.88/7.19557
3m	4-Cl	4-Br	(*R*)-CH_3	2	4	16	16	4	8	8	8	16	16	0.7527	5.53 ± 0.54	5.47/5.60525
3n	4-Cl	4-Br	(*S*)-CH–$(CH_3)_2$	4	4	8	16	4	8	8	8	8	8	0.9803	6.41 ± 0.54	6.36/6.53325
3o	4-Cl	4-Br	(*R*)-CH–$(CH_3)_2$	2	4	16	16	4	8	8	8	8	8	0.9805	6.41 ± 0.54	6.36/6.53325
3p	4-Cl	4-Br	(*S*)-CH_2-phenyl	4	8	16	16	4	8	8	16	16	16	1.1258	7.46 ± 0.55	7.15/7.02325
3q	4-Cl	4-Br	(*R*)-CH_2-phenyl	2	4	8	16	2	4	4	8	8	8	1.1267	7.46 ± 0.55	7.15/7.02325
3r	4-Cl	4,3-diCl	H	1	2	16	32	8	8	16	8	8	8	0.8639	5.91 ± 0.50	5.27/5.7738
3s	4-Cl	4,3-diCl	(*S*)-CH_3	2	4	16	32	4	4	8	8	16	16	0.9408	6.26 ± 0.50	5.76/6.0828
3t	4-Cl	4,3-diCl	(*R*)-CH_3	2	4	16	32	8	8	16	4	8	8	0.9380	6.26 ± 0.50	5.76/6.0828
3u	4-Cl	4,3-diCl	(*S*)-CH–$(CH_3)_2$	2	4	16	32	4	4	8	8	8	8	1.1567	7.14 ± 0.50	6.65/7.0108
3v	4-Cl	4,3-diCl	(*R*)-CH–$(CH_3)_2$	2	4	16	16	4	4	8	8	8	16	1.1559	7.14 ± 0.50	6.65/7.0108
3w	4-Cl	4,3-diCl	(*S*)-CH_2-phenyl	2	2	16	32	4	4	8	8	8	8	1.3056	8.19 ± 0.51	7.44/7.5008
3x	4-Cl	4,3-diCl	(*R*)-CH_2-phenyl	2	2	16	32	4	4	8	8	8	8	1.3030	8.19 ± 0.51	7.44/7.5008
3y	5-Cl	4-Cl	(*S*)-CH_3	4	8	16	16	4	8	8	8	8	16	0.6337	5.63 ± 0.48	5.20/5.45525
3z	5-Cl	4-Cl	(*R*)-CH_3	4	8	16	16	8	8	8	4	8	16	0.6334	5.63 ± 0.48	5.20/5.45525
3aa	5-Cl	4-Cl	(*S*)-CH–$(CH_3)_2$	4	8	16	16	4	8	8	4	8	16	0.8804	5.61 ± 0.48	6.09/6.38325
3bb	5-Cl	4-Cl	(*R*)-CH–$(CH_3)_2$	4	4	8	16	4	8	8	4	8	8	0.8816	5.61 ± 0.48	6.09/6.38325
3cc	5-Cl	4-Cl	(*S*)-CH_2-phenyl	4	4	8	8	4	8	8	4	8	8	1.0304	7.56 ± 0.49	6.88/6.87325
3dd	5-Cl	4-Cl	(*R*)-CH_2-phenyl	4	4	8	8	4	8	8	4	8	8	1.0315	7.56 ± 0.49	6.88/6.87325
INH				0.5	0.5	>250	>250	>250	>250	>250	4	8	8			

Table 2
Rate constants and half-times of the most active compounds in phosphate buffer solution pH 7.4 and pH 5.5

Compd	Rate constant k_{obs} (s^{-1}) pH 7.4	$t_{1/2}$ (s) for pH 7.4	Rate constant k_{obs} (s^{-1}) pH 5.5	$t_{1/2}$ (s) for pH 5.5	EC_{50} (μM)	SI for *M. tbc*
3n	7.79×10^{-4}	889.94	8.06×10^{-4}	860.1	121.8	16.74
3o	7.70×10^{-4}	897.47	7.81×10^{-4}	887.25	121.3	33.35
3q	3.37×10^{-4}	2057.7	3.56×10^{-4}	1948.5	35.5	10.61
3bb	5.65×10^{-4}	1226.10	5.69×10^{-4}	1218.1	106.7	13.75
3cc	4.56×10^{-4}	1520.80	5.29×10^{-4}	1311.5	82.94	20.73
3dd	5.24×10^{-4}	1323.30	4.35×10^{-4}	1074.0	104.2	26.05

lysis half-time in the range of 14–34 min should be sufficient for the release of active molecules at the site of action.

7. Quantitative structure–activity relationships

7.1. QSARs analyses

For 30 *N*-Cbz-amino acid esters **3** QSAR analyses in Trajan 6.0[23] were carried out. Relationships between the antimycobacterial activities against *M. tuberculosis* 331/88; *M. avium* 330/88; *M. kansasii* 235/80; *M. kansasii* 6509/96 (i.e., log MIC) after 21 days of incubation and 17 descriptors of the compounds were sought. Free-Wilson binary presence/absence descriptors (of substituents in R^1, R^2, and R^3), experimentally determined log *k*, Hammett substituent σ constants (for substituents in R^1 and R^2),[24] and Taft inductive σ^* constants (for substituents in R^3)[25] were used as descriptors. These descriptors are able to express lipophilic, electronic, and steric properties of the structures studied and can be easily attainable from the formula or from the literature.

According to sensitivity analysis of QSAR models for *N*-Cbz-amino acid esters **3**, Free-Wilson descriptor of R^2 = 3-Cl was found as the most important statistically for the activity against *M. tuberculosis* 331/88, Free-Wilson descriptor of R^2 = 4-Cl, for the activity against *M. avium* 330/88, Free-Wilson descriptor of R^2 = 4-Br, for the activity against *M. kansasii* 235/80, and Free-Wilson descriptor of R^2 = 3-Cl, for the activity against M. kansasii 6509/96. Substituents in R^2 of *N*-Cbz-amino acid esters **3** seem to have a greater influence on the activity than those in R^1 or R^3. Log *k* of *N*-Cbz-amino acid esters **3** appears to be of higher importance only for the activity against *M. kansasii* 235/80.

Description and details of used QSAR method, characterization of artificial neural networks, and the assessed results are provided in the Supplementary data.

8. SAR and QSAR

The results of in vitro evaluation showed an interesting activity for all compounds. Cbz-Amino acid esters **3** have exhibited an activity comparable with isoniazid (INH) as an internal standard against M. tuberculosis. Activities against *M. avium* and *M. kansasii* exceed the activity of INH considerably.

Compounds **3r**, **3w/x**, **3m**, **3o**, **3q,** and **3s–3v** exhibited the highest antituberculotic activity against M. tuberculosis. Based on these observations it can be concluded that both substitutions R^1 = 4-Cl and R^2 = 3,4-Cl or 4-Br are necessary for high activity while substitution of R^3 rather modulates lipophilicity that was found to be a secondary parameter with the range of log k 0.94–1.16. Based on the QSAR results the substituent in R^2 was selected as the determining factor for the change of anti-TB activity against *M. tuberculosis*.

Compounds **3cc**/**dd**, **3bb**, **3q,** and **3n** showed the highest activity against M. avium. From this fact it can be assumed that substitutions of both $R^1 = R^2$ in the $C_{(4)}$ positions are strongly important for high activity as well as substitution of R^3 by branched substituent (benzyl or isopropyl—radical analogy).

Compounds **3q**, **3u**, **3w/x,** and **3bb**–**3dd** showed the highest antimycobacterial activity against both evaluated strains of M. kansasii. It means that both substitutions R^1 = 4-Cl and R^2 = 4-Br or 3,4-Cl in $C_{(4)}$ positions are recommended for high activity. Furthermore it can be specified that R^1 = 5-Cl is a more advantageous substitution for higher active compounds against *M. kansasii* 6509/96. The substitution of R^3 by branched substituent (benzyl or isopropyl) is also connected with the activity (radical analogy). Finally, lipophilicity is another important parameter, the lipophilicity range is 0.88–1.16, and the optimum is about 1.1. The substituents R^1 and R^2 are relevant for the change of antituberculotic activity in accordance with the results of QSAR analysis.

A very important parameter influencing the activity is stereoisomerism, because individual enantiomers demonstrate considerable difference in their antituberculosis/antimycobacterial activity, for example, (*S*)-enantiomer **3p** showed much lower antituberculosis activity than (*R*)-enantiomer **3q** and enantiomers **3n**/**o** and **3u**/**v** showed different antimycobacterial activity. The determined differences in antituberculosis/antimycobacterial activity for individual *R*/*S*-enantiomers cannot be explained on the basis of the results presented here. Due to these facts it can be assumed that stereospecific bond can be probably formed between the compound and an enzyme in *Mycobacterium* sp. with subsequent enzyme inhibition.

9. Conclusion

Esterification of active molecules is one of the most frequent structural modifications that lead to better bioavailability. Lipophilic simple amino acids were chosen as natural molecules with increased lipophilicity and consequently permeability through cell membranes. The life-time of the prepared esters seems to be convenient for transport to the target. All 30 salicylanilide esters showed high antimycobacterial activity (comparable with or higher than the standard) against all tested mycobacterial strains and the selected six derivatives showed selectivity index higher than ten. The present study encourages further research of the series of compounds in order to find a new and novel antitubercular drug that would also be active in MDR cases. Structure analyses relationship is in accordance with QSAR results.

10. Experimental

10.1. Chemistry

10.1.1. Instrumentation and chemicals

The chemicals were purchased from commercial sources (Sigma–Aldrich, Merck). Commercial grade reagents were used without further purification. Reactions were monitored by means of thin layer chromatography plates coated with 0.2 mm Silica Gel 60 F254 (Merck). TLC plates were visualized by UV irradiation (254 nm). The products were purified by crystallization, by means of column flash chromatography employing Silica Gel 60 (Merck). All melting points were determined on Melting Point B-545 apparatus (Büchi, Germany) and are uncorrected. Optical activities were measured on polarimeter ADP 220 BS (Bellingham Stanley Ltd). Infrared spectra (KBr pellets) were recorded on FT-IR spectrometer Nicolet 6700 FT-IR in the range of 4000–400 cm^{-1}. NMR spectra were measured in $CDCl_3$ or DMSO-d_6 solutions (if not specified otherwise) on Varian Mercury—Vxbb 300 (300 MHz for ^1H and 75.5 MHz for ^{13}C; Varian Comp. Palo Alto, CA, USA). The chemical shifts δ are given in ppm, relating to tetramethylsilane (TMS) as an internal standard. The coupling constants (*J*) are reported in hertz. Elemental analyses (C, H, N) were performed on automatic microanalyzer CHNS–O CE Instrument (FISONS EA 1110, Milano, Italy).

10.1.2. Procedures and data of the prepared target compounds

10.1.2.1. General procedure for preparation of esters of Cbz-α-amino acids and substituted salicylanilides 3. *N*-Benzyloxycarbonyl (Cbz) protected 2-amino acid **2** (10 mmol) and substituted salicylanilide **1** (10 mmol) were dissolved in dry *N,N*-dimethylformamide (DMF, 45 mL). The solution was cooled to −10 °C and *N,N′*-dicyclohexylcarbodiimide (DCI, 11 mmol) was added in three portions during 1 h. The mixture was then stirred for 3 h at the same temperature and stored at +4 °C for 20 h. The precipitate of *N,N′*-dicyclohexylurea was removed by filtration and the solvent was evaporated in vacuo. The crude product **3** was purified by crystallization from ethyl acetate–hexane.

10.1.2.2. Data of prepared derivatives 3. 4-Chloro-2-(3-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)acetate (**3a**).[10]

(*S*)-4-Chloro-2-(3-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)propanoate (**3b**).[10]

(*R*)-4-Chloro-2-(3-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)propanoate (**3c**).[10]

(S)-4-Chloro-2-(3-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)-3-methylbutanoate (**3d**). White solid; yield 47%; mp 149–151 °C; $[\alpha]_D^{25}$ −30.7 (*c* 2.1, $CHCl_3$), $[\alpha]_D^{23}$ −86.0 (*c* 1.4, ethyl acetate). IR (KBr pellet): 3330, 2965, 1764 (CO ester), 1712, 1667, 1592, 1532, 1481, 1424, 1310, 1199, 1104, 896, 781, 752, 700, 530 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6) δ 8.21 (1H, br s, NH), 7.82–7.72 (2H, m, H3, H2′), 7.53–7.40 (2H, m, H5, H6′), 7.36–7.28 (4H, m, H5′, H3″, H4″, H5″), 7.28–7.20 (2H, m, H2″, H6″), 7.16–7.09 (2H, m, H4′, H6), 5.28 (1H, d, *J* = 8.0 Hz, NH), 5.11 (1H, d, *J* = 12.1 Hz, OCH2), 5.02 (1H, d, *J* = 12.1 Hz, OCH_2), 4.39 (1H, dd, *J* = 8.0 Hz, *J* = 5.2 Hz, CH), 2.37–2.17 (1H, m, CH), 1.03 (3H, d, *J* = 6.9 Hz, CH_3), 0.93 (3H, d, *J* = 6.9 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.6, 162.8, 156.4, 145.6, 138.6, 135.8, 134.6, 132.2, 132.0, 130.0, 129.9, 129.8, 128.6, 128.3, 128.2, 124.9, 124.3, 120.7, 118.5, 67.4, 59.7, 30.4, 19.2, 17.6. Anal. Calcd for $C_{26}H_{24}Cl_2N_2O_5$ (515.40): C, 60.59; H, 4.69; N, 5.44. Found: C, 60.40; H, 4.99; N, 5.57.

(R)-4-Chloro-2-(3-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)-3-methylbutanoate (**3e**). White solid; yield 34%; mp 159–162 °C; $[\alpha]_D^{26}$ 18.7 (*c* 2.4, $CHCl_3$). IR (KBr pellet): 3332, 2964, 1764 (CO ester), 1734, 1712, 1667, 1592, 1533, 1480, 1423, 1310, 1202, 1104, 1038, 896, 782, 753, 586, 531 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6) δ 8.21 (1H, br s, NH), 7.82–7.72 (2H, m, H3, H2′), 7.53–7.40 (2H, m, H5, H6′), 7.36–7.28 (4H, m, H5′, H3″, H4″, H5″), 7.28–7.20 (2H, m, H2″, H6″), 7.16–7.09 (2H, m, H6, H4′), 5.28 (1H, d, *J* = 8.0 Hz, NH), 5.11 (1H, d, *J* = 12.0 Hz, OCH_2), 5.02 (1H, d, *J* = 12.1 Hz, OCH_2), 4.39 (1H, dd, *J* = 8.0 Hz, *J* = 5.2 Hz, CH), 2.37–2.17 (1H, m, CH), 1.03 (3H, d, *J* = 6.9 Hz, CH_3), 0.93 (3H, d, *J* = 7.0 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.7, 162.3, 156.4, 145.6, 138.6, 135.8, 134.6, 132.2, 132.0, 130.0, 129.9,

129.8, 128.6, 128.3, 128.2, 124.9, 124.3, 120.7, 118.5, 67.4, 59.7, 30.4, 19.2, 17.6. Anal. Calcd for $C_{26}H_{24}Cl_2N_2O_5$ (515.40): C, 60.59; H, 4.69; N, 5.44. Found: C, 60.59; H, 5.09; N, 5.78.

(S)-4-Chloro-2-(3-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)-3-phenylpropanoate (**3f**). White solid; yield 33%; mp 157–159 °C; $[\alpha]_D^{26}$ −12.2 (*c* 1.8, $CHCl_3$). IR (KBr pellet): 3326, 2929, 2851, 1761 (CO ester), 1705, 1658, 1627, 1594, 1538, 1483, 1424, 1311, 1262, 1200, 1161, 1106, 1082, 1054, 1029, 779, 698, 682 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 8.25 (1H, br s, NH), 7.80 (1H, d, *J* = 2.1 Hz, H3), 7.73 (1H, m, H2′), 7.49–7.46 (1H, m, H4′), 7.41 (1H, dd, *J* = 8.5 Hz, *J* = 2.1 Hz, H5), 7.32–7.27 (10H, m, H6′, H5′, Ar-phenyl), 7.22–7.16 (2H, m, Ar-phenyl), 6.93 (1H, d, *J* = 8.5 Hz, H6), 5.27 (1H, d, *J* = 7.2 Hz, NH), 5.06 (1H, d, *J* = 12.3 Hz, OCH_2), 4.99 (1H, d, *J* = 12.3 Hz, OCH_2), 4.75 (1H, q, *J* = 6.9 Hz, NCH), 3.24 (1H, dd, *J* = 13.9 Hz, *J* = 6.3 Hz, CH_2), 3.10 (1H, dd, *J* = 13.9 Hz, *J* = 7.5 Hz, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 162.2, 155.9, 145.7, 138.6, 135.7, 134.9, 134.5, 132.2, 132.1, 130.0, 129.9, 129.4, 129.2, 128.9, 128.5, 128.3, 128.1, 127.5, 124.9, 124.3, 120.8, 118.7, 67.4, 55.4, 37.3. Anal. Calcd for $C_{30}H_{24}Cl_2N_2O_5$ (563.43): C, 63.95; H, 4.29; N, 4.97. Found: C, 64.35; H, 4.50; N, 5.32.

(R)-4-Chloro-2-(3-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)-3-phenylpropanoate (**3g**). White solid; yield 48%; mp 161–163 °C; $[\alpha]_D^{26}$ 19.1 (*c* 2.9, $CHCl_3$). IR (KBr pellet): 3299, 3064, 3033, 1761 (CO ester), 1705, 1658, 1594, 1538, 1483, 1424, 1306, 1262, 1200, 1161, 1148, 1106, 1081, 1054, 883, 779, 698, 682 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 8.20 (1H, br s, NH), 7.76 (1H, d, *J* = 2.4 Hz, H3), 7.73 (1H, m, H2′), 7.48–7.45 (1H, m, H4′), 7.41 (1H, dd, *J* = 8.7 Hz, *J* = 2.4 Hz, H5), 7.33–7.28 (10H, m, H6′, H5′, Ar-phenyl), 7.23–7.10 (2H, m, Ar-phenyl), 6.93 (1H, d, *J* = 8.7 Hz, H6), 5.25 (1H, d, *J* = 7.2 Hz, NH), 5.06 (1H, d, *J* = 12.0 Hz, OCH_2), 4.99 (1H, d, *J* = 12.3 Hz, OCH_2), 4.75 (1H, q, *J* = 6.6 Hz, NCH), 3.24 (1H, dd, *J* = 14.0 Hz, *J* = 6.2 Hz, CH_2), 3.10 (1H, dd, *J* = 14.0 Hz, *J* = 7.7 Hz, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 162.2, 155.9, 145.7, 138.6, 135.7, 134.9, 134.5, 132.3, 132.1, 130.0, 129.9, 129.4, 129.1, 128.9, 128.5, 128.3, 128.1, 127.5, 125.0, 124.3, 120.8, 118.7, 67.4, 55.4, 37.3. Anal. Calcd for $C_{30}H_{24}Cl_2N_2O_5$ (563.43): C, 63.95; H, 4.29; N, 4.97. Found: C, 64.15; H, 4.60; N, 5.09.

(R)-4-Chloro-2-(4-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)propanoate (**3h**). White solid; yield 12%; mp 137–139 °C; $[\alpha]_D^{25}$ 37.0 (*c* 2.3, $CHCl_3$). IR (KBr pellet): 3307, 1768 (CO ester), 1698, 1657, 1538, 1533, 1493, 1455, 1404, 1315, 1262, 1196, 1101, 1065, 825, 736, 697, 508 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 8.13 (1H, br s, NH), 7.77 (1H, m, H3), 7.56–7.28 (10H, m, H2′, H6′, H4, H3′, H5′, H2″, H3″, H4″, H5″, H6″), 7.11 (1H, d, *J* = 8.7 Hz, H6), 5.30 (1H, d, *J* = 6.9 Hz, NH), 5.11 (1H, d, *J* = 12.0 Hz, OCH_2), 5.04 (1H, d, *J* = 12.0 Hz, OCH_2), 4.54 (1H, m, NCH), 1.48 (3H, d, *J* = 7.2 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.4, 162.2, 155.8, 145.8, 136.0, 135.9, 132.3, 132.1, 130.0, 129.9, 129.7, 129.0, 128.6, 128.3, 128.1, 124.5, 121.7, 67.3, 50.1, 17.5. Anal. Calcd for $C_{24}H_{20}Cl_2N_2O_5$ (487.33): C, 59.19; H, 4.14; N, 5.75. Found: C, 59.02; H, 4.43; N, 5.85.

(S)-4-Chloro-2-(4-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)-3-methylbutanoate (**3i**).[10]

(R)-4-Chloro-2-(4-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)-3-methylbutanoate (**3j**).[10]

(S)-4-Chloro-2-(4-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)-3-phenylpropanoate (**3k**).[10,19]

(R)-4-Chloro-2-(4-chlorophenylcarbamoyl)phenyl2-(benzyloxycarbonylamino)-3-phenylpropanoate (**3l**). White solid; yield 46%; mp 163–166 °C; $[\alpha]_D^{25}$ 18.4 (*c* 3.5, $CHCl_3$). IR (KBr pellet): 3226, 2922, 2851, 1762 (CO ester), 1706, 1663, 1597, 1534, 1493, 1403, 1313, 1260, 1198, 1147, 1101, 826, 751, 699 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 8.14 (1H, br s, NH), 7.81 (1H, d, *J* = 2.5 Hz, H3), 7.55–7.53 (2H, m, H2′, H6′), 7.40 (1H, dd, *J* = 8.5 Hz, *J* = 2.5 Hz, H5), 7.36–7.21 (10H, m, H3′, H5′, Ar-phenyl), 7.19–7.11 (2H, m, Ar-phenyl), 6.93 (1H, d, *J* = 8.7 Hz, H6), 5.25 (1H, d, *J* = 7.2 Hz, NH), 5.04 (1H, d, *J* = 12.0 Hz, OCH_2), 4.97 (1H, d, *J* = 12.3 Hz, OCH_2), 4.75 (1H, q, *J* = 7.2 Hz, NCH), 3.22 (1H, dd, *J* = 13.5 Hz, *J* = 6.4 Hz, CH_2), 3.10 (1H, dd, *J* = 13.5 Hz, *J* = 7.4 Hz, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.1, 162.1, 155.9, 145.7, 135.9, 13,5.8, 134.9, 132.3, 132.1, 130.0, 129.5, 129.2, 129.0, 128.9, 128.6, 128.4, 128.3, 128.1, 127.6, 124.3, 121.9, 67.4, 55.4, 37.4. Anal. Calcd for $C_{30}H_{24}Cl_2N_2O_5$ (563.43): C, 63.95; H, 4.29; N, 4.97. Found: C, 64.285; H, 4.57; N, 5.13.

(R)-2-(4-Bromophenylcarbamoyl)-4-chlorophenyl 2-(benzyloxycarbonylamino)propanoate (**3m**). White solid; yield 3%; mp 190–192 °C; $[\alpha]_D^{25}$ 38.1 (*c* 1.8, $CHCl_3$). IR (KBr pellet): 3313, 2935, 1767 (CO ester), 1697, 1657, 1533, 1489, 1455, 1403, 1314, 1262, 1196, 1166, 1102, 1071, 1010, 882, 822, 736, 696, 506 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 8.15 (1H, br s, NH), 7.76 (1H, m, H3), 7.52–7.33 (10H, m, H5, H2′, H6′, H3′, H5′, H2″, H3″, H4″, H5″, H6″), 7.11 (1H, d, *J* = 8.7 Hz, H6), 5.31 (1H, d, *J* = 7.2 Hz, NH), 5.11 (1H, d, *J* = 12.3 Hz, OCH_2), 5.03 (1H, d, *J* = 12.3 Hz, OCH_2), 4.53 (1H, m, NCH), 1.48 (3H, d, *J* = 7.2 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.4, 162.2, 155.8, 145.8, 136.5, 135.6, 132.3, 132.1, 132.0, 129.7, 128.6, 128.3, 128.1, 124.5, 122.0, 117.6, 67.3, 50.1, 33.8, 17.5. Anal. Calcd for $C_{24}H_{20}BrClN_2O_5$ (531.78): C, 54.21; H, 3.79; N, 5.27. Found: C, 54.28; H, 3.92; N, 5.42.

(S)-2-(4-Bromophenylcarbamoyl)-4-chlorophenyl 2-(benzyloxycarbonylamino)-3-methylbutanoate (**3n**). White solid; yield 48%; mp 144–146 °C; $[\alpha]_D^{26}$ −24.3 (*c* 2.2, $CHCl_3$). IR (KBr pellet): 3308, 2960, 1748 (CO ester), 1707, 1668, 1531, 1489, 1394, 1315, 1252, 1189, 1105, 1069, 1008, 815, 744, 695 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6) δ 8.17 (1H, br s, NH), 7.82–7.77 (1H, m, H3), 7.54–7.28 (10H, m, H5, H2′, H3′, H5′, H6′, H2″, H3″, H4″, H5″, H6″), 7.12 (1H, d, *J* = 8.8 Hz, H6), 5.27 (1H, d, *J* = 8.2 Hz, NH), 5.10 (1H, d, *J* = 12.4 Hz, OCH_2), 5.01 (1H, d, *J* = 12.4 Hz, OCH_2), 4.39 (1H, dd, *J* = 8.2 Hz, *J* = 5.5 Hz, CH), 2.35–2.18 (1H, m, CH), 1.03 (3H, d, *J* = 6.9 Hz, CH_3), 0.93 (3H, d, *J* = 6.9 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.5, 162.2, 156.4, 145.6, 136.5, 135.8, 132.2, 132.0, 131.9, 130.0, 129.8, 128.6, 128.4, 128.1, 124.3, 122.1, 117.6, 67.4, 59.7, 30.5, 19.2, 17.6. Anal. Calcd for $C_{26}H_{24}BrClN_2O_5$ (559.83): C, 55.78; H, 4.32; N, 5.00. Found: C, 56.065, H, 4.565; N, 4.97.

(R)-2-(4-Bromophenylcarbamoyl)-4-chlorophenyl 2-(benzyloxycarbonylamino)-3-methylbutanoate (**3o**). White solid; yield 32%; mp 147–150 °C; $[\alpha]_D^{26}$ 16.4 (*c* 2.1, $CHCl_3$). IR (KBr pellet): 3331, 2964, 1763 (CO ester), 1705, 1669, 1601, 1533, 1489, 1394, 1315, 1253, 1199, 1105, 1070, 1008, 827, 696, 507 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 8.18 (1H, br s, NH), 7.79 (1H, m, H3), 7.52–7.32 (10H, m, H5, H2′, H6′, H3′, H5′, H2″, H3″, H4″, H5″, H6″), 7.12 (1H, d, *J* = 8.7 Hz, H6), 5.28 (1H, d, *J* = 8.4 Hz, NH), 5.10 (1H, d, *J* = 12.3 Hz, OCH_2), 5.00 (1H, d, *J* = 12.0 Hz, OCH_2), 4.39 (1H, dd, *J* = 4.8 Hz, *J* = 1.4 Hz, NCH), 2.30–2.23 (1H, m, CH), 1.03 (3H, d, *J* = 6.9 Hz, CH_3), 0.93 (3H, d, *J* = 6.9 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.5, 162.2, 156.4, 145.6, 136.5, 135.8, 132.2, 132.0, 131.9, 130.0, 129.8, 128.6, 128.4, 128.1, 124.3, 122.1, 117.6, 67.4, 59.7, 30.5, 19.2, 17.6. Anal. Calcd for $C_{26}H_{24}BrClN_2O_5$ (559.84): C, 55.78; H, 4.32; N, 5.00. Found: C, 55.79; H, 4.61; N, 5.11.

(S)-2-(4-Bromophenylcarbamoyl)-4-chlorophenyl 2-(benzyloxycarbonylamino)-3-phenylpropanoate (**3p**). White solid; yield 32%; mp 168–170 °C; $[\alpha]_D^{26}$ −15.2 (*c* 1.65, $CHCl_3$). IR (KBr pellet): 3312, 1761 (CO ester), 1704, 1659, 1593, 1533, 1489, 1456, 1402, 1313, 1261, 1199, 1101, 1050, 1010, 820, 750, 698, 504 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 8.15 (1H, br s, NH), 7.80 (1H, d, *J* = 2.4 Hz, H3), 7.49 (2H, m, H2′, H6′), 7.50–7.31 (11H, m, H5, H3′, H5′, Ar-phenyl), 7.16 (2H, m, Ar-phenyl), 6.92 (1H, d, *J* = 8.7 Hz, H6), 5.25 (1H, d, *J* = 6.9 Hz, NH), 5.04 (1H, d, *J* = 12.3 Hz, OCH_2), 4.96 (1H, d, *J* = 12.3 Hz, OCH_2), 4.75 (1H, q, *J* = 6.9 Hz, NCH), 3.23 (1H, dd, *J* = 14.0 Hz, *J* = 6.2 Hz, CH_2), 3.10 (1H, dd, *J* = 14.0 Hz, *J* = 7.5 Hz, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.1, 162.1, 155.9, 145.7,

136.5, 135.7, 134.9, 132.3, 132.1, 131.9, 130.0, 129.4, 129.2, 128.9, 128.6, 128.3, 128.1, 127.6, 124.3, 122.3, 117.6, 67.4, 55.3, 37.4. Anal. Calcd for $C_{30}H_{24}BrClN_2O_5$ (607.88): C, 59.28; H, 3.98; N, 4.61. Found: C, 59.23; H, 4.00; N, 4.60.

(R)-2-(4-Bromophenylcarbamoyl)-4-chlorophenyl 2-(benzyloxycarbonylamino)-3-phenylpropanoate (**3q**). White solid; yield 37%; mp 169–171 °C; $[\alpha]_D^{26}$ 17.8 (*c* 0.9, $CHCl_3$). IR (KBr pellet): 3309, 1762 (CO ester), 1704, 1659, 1533, 1489, 1455, 1401, 1313, 1261, 1198, 1140, 1104, 1029, 1010, 822, 750, 698 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 8.19 (1H, br s, NH), 7.79 (1H, d, J = 2.4 Hz, H3), 7.48 (2H, m, H2′, H6′), 7.50–7.28 (11H, m, H5, H3′, H5′, Ar-phenyl), 7.16 (2H, m, Ar-phenyl), 6.92 (1H, d, J = 8.7 Hz, H6), 5.27 (1H, d, J = 7.2 Hz, NH), 5.04 (1H, d, J = 12.3 Hz, OCH_2), 4.96 (1H, d, J = 12.3 Hz, OCH_2), 4.75 (1H, q, J = 7.2 Hz, NCH), 3.23 (1H, dd, J = 14.0 Hz, J = 6.2 Hz, CH_2), 3.10 (1H, dd, J = 14.0 Hz, J = 7.2 Hz, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.1, 162.1, 155.9, 145.7, 136.5, 135.7, 134.9, 132.2, 132.1, 131.9, 130.0, 129.5, 129.2, 128.9, 128.6, 128.3, 128.1, 127.5, 124.3, 122.3, 117.6, 67.4, 55.3, 37.4. Anal. Calcd for $C_{30}H_{24}BrClN_2O_5$ (607.88): C, 59.28; H, 3.98; N, 4.61. Found: C, 59.08; H, 4.28; N, 4.70.

4-Chloro-2-(3,4-dichlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)acetate (**3r**).[10]

(S)-4-Chloro-2-(3,4-dichlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)propanoate (**3s**). White solid; yield 13%; mp 136–138 °C; $[\alpha]_D^{26}$ −36.1 (*c* 1.7, $CHCl_3$). IR (KBr pellet): 3305, 1768 (CO ester), 1700, 1659, 1589, 1525, 1477, 1454, 1381, 1307, 1257, 1199, 1103, 1065, 1028, 881, 818, 736, 697 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$) δ 8.32 (1H, br s, NH), 7.86–7.75 (2H, m, H3, H2′), 7.48–7.44 (2H, m, H5, H6′), 7.35–7.33 (6H, m, H5′, H2″, H3″, H4″, H5″, H6″), 7.10 (1H, d, J = 8.7 Hz, H6), 5.32 (1H, d, J = 6.9 Hz, NH), 5.12 (1H, d, J = 12.0 Hz, OCH_2), 5.02 (1H, d, J = 12.0 Hz, OCH_2), 4.51 (1H, m, NCH), 1.49 (3H, d, J = 7.2 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.4, 162.3, 155.9, 145.8, 136.9, 135.8, 132.7, 132.2, 131.0, 130.4, 129.9, 129.2, 128.6, 128.3, 128.1, 127.0, 124.4, 122.4, 119.9, 67.4, 50.1, 17.3. Anal. Calcd for $C_{24}H_{19}Cl_3N_2O_5$ (521.78): C, 55.25; H, 3.67; N, 5.37. Found: C, 55.15; H, 3.75; N, 5.44.

(R)-4-Chloro-2-(3,4-dichlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)propanoate (**3t**). White solid; yield 4%; mp 134–136 °C; $[\alpha]_D^{25}$ 34.5 (*c* 2.0, $CHCl_3$). IR (KBr pellet): 3305, 1768, 1699, 1659, 1589, 1526, 1477, 1455, 1381, 1300, 1261, 1200, 1103, 1067, 1029, 881, 819, 736, 697 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 8.30 (1H, br s, NH), 7.87–7.77 (2H, m, H3, H2′), 7.48–7.32 (8H, m, H5, H5′, H6′, H2″, H3″, H4″, H5″, H6″), 7.10 (1H, d, J = 8.7 Hz, H6), 5.30 (1H, d, J = 6.6 Hz, NH), 5.11 (1H, d, J = 12.0 Hz, OCH_2), 5.02 (1H, d, J = 12.3 Hz, OCH_2), 4.52 (1H, m, NCH), 1.50 (3H, d, J = 7.2 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.4, 162.3, 156.0, 145.8, 137.0, 135.8, 132.3, 130.4, 130.0, 129.7, 129.2, 128.6, 128.4, 128.1, 127.0, 126.7, 124.4, 122.4, 119.9, 67.4, 50.1, 17.3. Anal. Calcd for $C_{24}H_{19}Cl_3N_2O_5$ (521.78): C, 55.25; H, 3.67; N, 5.37. Found: C, 55.575; H, 3.815; N, 5.52.

(S)-4-Chloro-2-(3-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)-3-methylbutanoate (**3u**). White solid; yield 33%; mp 144–147 °C; $[\alpha]_D^{26}$ −20.9 (*c* 1.5, $CHCl_3$). IR (KBr pellet): 3324, 2966, 1763 (CO ester), 1706, 1669, 1587, 1523, 1477, 1382, 1308, 1200, 1104, 1027, 820, 745, 698, 578 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6) δ 8.32 (1H, br s, NH), 7.88 (1H, d, J = 2.0 Hz, H3), 7.81 (1H, d, J = 2.2 Hz, H2′), 7.52–7.41 (2H, m, H5, H5′), 7.38–7.29 (6H, m, H6′, H2″, H3″, H4″, H5″, H6), 7.13 (1H, d, J = 8.5 Hz, H6), 5.27 (1H, d, J = 7.7 Hz, NH), 5.12 (1H, d, J = 12.2 Hz, OCH_2), 5.02 (1H, d, J = 12.2 Hz, OCH_2), 4.35 (1H, dd, J = 7.7 Hz, J = 5.5 Hz, CH), 2.36–2.19 (1H, m, CH), 1.06 (3H, d, J = 6.9 Hz, CH_3), 0.97 (3H, d, J = 6.9 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.5, 162.3, 156.5, 145.6, 137.0, 135.7, 134.4, 132.6, 132.3, 132.2, 130.4, 130.2, 129.3, 128.6, 128.4, 128.1, 124.3, 122.5, 120.0, 67.5, 59.9, 30.4, 19.2, 17.8. Anal. Calcd for $C_{26}H_{23}Cl_3N_2O_5$ (549.83): C, 56.80; H, 4.22; N, 5.09. Found: C, 56.74; H, 4.56; N, 5.14.

(R)-4-Chloro-2-(3,4-dichlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)-3-methylbutanoate (**3v**). White solid; yield 15%; mp 150–152 °C; $[\alpha]_D^{26}$ 19.5 (*c* 2.7, $CHCl_3$). IR (KBr pellet): 3324, 2967, 1767 (CO ester), 1704, 1670, 1588, 1525, 1477, 1382, 1308, 1202, 1104, 1028, 821, 698 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6) δ 8.32 (1H, br s, NH), 7.88 (1H, d, J = 2.0 Hz, H3), 7.81 (1H, d, J = 2.2 Hz, H2′), 7.52–7.41 (2H, m, H5, H5′), 7.38–7.29 (6H, m, H6′, H2″, H3″, H4″, H5″, H6″), 7.13 (1H, d, J = 8.5 Hz, H6), 5.27 (1H, d, J = 7.7 Hz, NH), 5.12 (1H, d, J = 12.2 Hz, OCH_2), 5.02 (1H, d, J = 12.2 Hz, OCH_2), 4.35 (1H, dd, J = 7.7 Hz, J = 5.5 Hz, CH), 2.36–2.19 (1H, m, CH), 1.06 (3H, d, J = 6.9 Hz, CH_3), 0.97 (3H, d, J = 6.9 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.5, 162.3, 156.5, 145.6, 137.0, 135.7, 134.4, 132.6, 132.3, 132.2, 130.4, 130.2, 129.3, 128.6, 128.4, 128.1, 124.3, 122.5, 120.0, 67.5, 59.9, 30.4, 19.2, 17.8. Anal. Calcd for $C_{26}H_{23}Cl_3N_2O_5$ (549.83): C, 56.80; H, 4.22; N, 5.09. Found: C, 57.20; H, 4.22; N, 5.12.

(S)-4-Chloro-2-(3,4-dichlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)-3-phenylpropanoate (**3w**). White solid; yield 27%; mp 169–172 °C; $[\alpha]_D^{26}$ −10.1 (*c* 2.1, $CHCl_3$). IR (KBr pellet): 3308, 1761 (CO ester), 1703, 1658, 1592, 1477, 1456, 1401, 1378, 1306, 1262, 1200, 1139, 1104, 1053, 1029, 1053, 1029, 883, 813, 698 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 8.33 (1H, br s, NH), 7.84 (1H, d, J = 1.9 Hz, H3), 7.80 (1H, d, J = 2.1 Hz, H2′), 7.46 (1H, dd, J = 8.7 Hz, J = 2.0 Hz, H5), 7.42 (1H, dd, J = 8.7 Hz, J = 2.4 Hz, H6′), 7.34–7.27 (9H, m, H5′, Ar-phenyl), 7.18 (2H, m, Ar-phenyl), 6.90 (1H, d, J = 8.7 Hz, H6), 5.27 (1H, d, J = 6.9 Hz, NH), 5.06 (1H, d, J = 12.3 Hz, OCH_2), 4.99 (1H, d, J = 12.3 Hz, OCH_2), 4.73 (1H, q, J = 7.2 Hz, NCH), 3.23 (1H, dd, J = 13.8 Hz, J = 6.3 Hz, CH_2), 3.10 (1H, dd, J = 13.8 Hz, J = 7.5 Hz, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 162.1, 156.0, 145.7, 136.9, 135.6, 134.7, 132.6, 132.3, 132.2, 130.4, 130.2, 129.8, 129.6, 129.1, 129.0, 128.6, 128.4, 128.1, 127.6, 124.4, 122.6, 120.2, 67.5, 55.4, 37.3. Anal. Calcd for $C_{30}H_{23}Cl_3N_2O_5$ (597.87): C, 60.27; H, 3.88; N, 4.69. Found: C, 60.59; H, 4.03; N, 4.82.

(R)-4-Chloro-2-(3,4-dichlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)-3-phenylpropanoate (**3x**). White solid; yield 10%; mp 167–170 °C; $[\alpha]_D^{26}$ 35.9 (*c* 1.6, $CHCl_3$). IR (KBr pellet): 3320, 2929, 2851, 1761 (CO ester), 1703, 1658, 1628, 1588, 1525, 1477, 1378, 1307, 1262, 1200, 1104, 1052, 1029, 814, 749, 698 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 8.32 (1H, br s, NH), 7.85 (1H, d, J = 2.1 Hz, H3), 7.80 (1H, d, J = 2.5 Hz, H2′), 7.47 (1H, dd, J = 8.7 Hz, J = 2.1 Hz, H5), 7.42 (1H, dd, J = 8.7 Hz, J = 2.4 Hz, H6′), 7.34–7.27 (9H, m, H5′, Ar-phenyl), 7.18 (2H, m, Ar-phenyl), 6.91 (1H, d, J = 8.7 Hz, H6), 5.26 (1H, d, J = 6.6 Hz, NH), 5.06 (1H, d, J = 12.3 Hz, OCH_2), 5.00 (1H, d, J = 12.3 Hz, OCH_2), 4.73 (1H, q, J = 7.2 Hz, NCH), 3.23 (1H, dd, J = 13.8 Hz, J = 6.3 Hz, CH_2), 3.10 (1H, dd, J = 13.8 Hz, J = 7.7 Hz, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 162.1, 156.0, 145.7, 137.0, 135.7, 134.8, 132.6, 132.3, 132.2, 130.4, 130.3, 129.9, 129.6, 129.2, 129.0, 128.6, 128.4, 128.1, 127.6, 124.3, 122.6, 120.2, 67.4, 55.4, 37.3. Anal. Calcd for $C_{30}H_{23}Cl_3N_2O_5$ (597.87): C, 60.27; H, 3.88; N, 4.69. Found: C, 60.65; H, 4.35; N, 5.01.

(S)-5-Chloro-2-(4-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)propanoate (**3y**). White solid; yield 57%; mp 157–160 °C; $[\alpha]_D^{26}$ −39.0 (*c* 1.7, $CHCl_3$). IR (KBr pellet): 3325, 1769 (CO ester), 1691, 1663, 1595, 1537, 1493, 1454, 1399, 1313, 1253, 1165, 1095, 1070, 1015, 910, 824, 738, 696, 508 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 8.19 (1H, br s, NH), 7.74 (1H, d, J = 8.4 Hz, H3), 7.65 (2H, m, H2′, H6′), 7.32–7.29 (8H, m, H4, H3′, H5′, H2″, H3″, H4″, H5″, H6″), 7.20 (1H, m, H6), 5.32 (1H, d, J = 6.9 Hz, NH), 5.12 (1H, d, J = 12.3 Hz, OCH_2), 5.04 (1H, d, J = 12.0 Hz, OCH_2), 4.53 (1H, m, NCH), 1.48 (3H, d, J = 7.2 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.1, 162.7, 155.8, 147.8, 137.7, 136.1, 135.8, 131.0, 129.9, 129.0, 128.6, 128.3, 128.1, 127.0, 126.7, 123.4, 121.8, 37.3, 50.1, 17.4. Anal. Calcd for $C_{24}H_{20}Cl_2N_2O_5$ (487.33): C, 59.15; H, 4.14; N, 5.75. Found: C, 59.23; H, 4.235; N, 5.78.

(R)-5-Chloro-2-(4-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)propanoate (**3z**). White solid; yield 21%; mp 155–157 °C; $[\alpha]_D^{26}$ 42.1 (*c* 1.65, $CHCl_3$), $[\alpha]_D^{23}$ 52.3 (*c* 1.1, ethyl acetate). IR (KBr pellet): 3325, 1769 (CO ester), 1691, 1663, 1595, 1538, 1494, 1454, 1399, 1313, 1267, 1166, 1095, 1071, 824, 738, 696, 507 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6) δ 8.23 (1H, br s, NH), 7.73 (1H, d, *J* = 8.2 Hz, H3), 7.61–7.49 (2H, m, H2′, H6′), 7.38–7.17 (9H, m, H4, H6, H3′, H5′, H2″, H3″, H4″, H5″, H6″), 5.36 (1H, d, *J* = 6.7 Hz, NH), 5.12 (1H, d, *J* = 12.1 Hz, OCH_2), 5.03 (1H, d, *J* = 12.1 Hz, OCH_2), 4.61–4.44 (1H, m, CH), 1.48 (3H, d, *J* = 7.2 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 171.1, 162.7, 155.9, 147.8, 137.7, 136.1, 135.8, 130.9, 129.8, 129.0, 128.6, 128.3, 128.1, 127.0, 126.7, 123.4, 121.8, 67.3, 50.1, 17.4. Anal. Calcd for $C_{24}H_{20}Cl_2N_2O_5$ (487.33): C, 59.15; H, 4.14; N, 5.75. Found: C, 59.42; H, 4.45; N, 5.89.

(S)-5-Chloro-2-(4-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)-3-methylbutanoate (**3aa**). White solid; yield 16%; mp 149–151 °C; $[\alpha]_D^{26}$ −25.5 (*c* 2.7, $CHCl_3$), $[\alpha]_D^{23}$ −37.5 (*c* 1.5, ethyl acetate). IR (KBr pellet): 3303, 2968, 1758 (CO ester), 1684, 1684, 1664, 1603, 1534, 1493, 1400, 1350, 1313, 1250, 1196, 1128, 1092, 1073, 1045, 820, 698, 508 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 8.23 (1H, br s, NH), 7.78 (1H, d, *J* = 8.4 Hz, H3), 7.58–7.55 (2H, m, H2′, H6′), 7.33–7.25 (8H, m, H4, H3′, H5′, H2″, H3″, H4″, H5″, H6″), 7.21 (1H, m H6), 5.29 (1H, d, *J* = 8.1 Hz, NH), 5.10 (1H, d, *J* = 12.3 Hz, OCH_2), 5.01 (1H, d, *J* = 12.0 Hz, OCH_2), 4.38 (1H, dd, *J* = 5.4 Hz, *J* = 2.7 Hz, NCH), 2.26 (1H, m, CH), 1.03 (3H, d, *J* = 6.9 Hz, CH_3), 0.93 (3H, d, *J* = 6.9 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.3, 162.6, 156.4, 147.6, 137.6, 136.1, 135.8, 131.3, 129.8, 128.9, 128.6, 128.4, 128.1, 127.0, 126.8, 123.3, 121.9, 67.4, 59.7, 30.5, 19.2, 17.6. Anal. Calcd for $C_{26}H_{24}Cl_2N_2O_5$ (515.40): C, 60.59; H, 4.69; N, 5.44. Found: C, 60.375; H, 4.97; N, 5.47.

(R)-5-Chloro-2-(4-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)-3-methylbutanoate (**3bb**). White solid; yield 42%; mp 148–150 °C; $[\alpha]_D^{26}$ 33.0 (*c* 1.3, $CHCl_3$). IR (KBr pellet): 3303, 2968, 1758 (CO ester), 1684, 1664, 1603, 1523, 1534, 1493, 1399, 1350, 1314, 1250, 1196, 1175, 1127, 1092, 1073, 1045, 1014, 827, 698, 659, 508 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 8.22 (1H, br s, NH), 7.78 (1H, d, *J* = 8.1 Hz, H3), 7.33–7.25 (10H, m, H4,H2′, H6′, H3′, H5′, H2″, H3″, H4″, H5″, H6″), 7.21 (1H, m, H6), 5.29 (1H, d, *J* = 8.1 Hz, NH), 5.10 (1H, d, *J* = 12.0 Hz, OCH_2), 5.01 (1H, d, *J* = 12.0 Hz, OCH_2), 4.38 (1H, dd, *J* = 5.4 Hz, *J* = 3.0 Hz, NCH), 2.29–2.23 (1H, m, CH), 1.04 (3H, d, *J* = 6.9 Hz, CH_3), 0.94 (3H, d, *J* = 6.9 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.3, 162.6, 156.4, 147.6, 137.6, 136.1, 135.8, 131.3, 129.8, 128.9, 128.6, 128.4, 128.1, 127.0, 126.8, 123.3, 121.9, 67.4, 59.7, 30.5, 19.2, 17.6. Anal. Calcd for $C_{26}H_{24}Cl_2N_2O_5$ (515.40): C, 60.59; H, 4.69; N, 5.44. Found: C, 60.67; H, 5.00; N, 5.43.

(S)-5-Chloro-2-(4-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)-3-phenylpropanoate (**3cc**). White solid; yield 42%; mp 185–187 °C; $[\alpha]_D^{25}$ −14.8 (*c* 1.8, $CHCl_3$). IR (KBr pellet): 3327, 1764 (CO ester), 1705, 1658, 1533, 1494, 1455, 1401, 1316, 1258, 1143, 1093, 1080, 1056, 826, 743, 699, 508 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 10.35 (1H, br s, NH), 8.02 (1H, d, *J* = 7.8 Hz, H3), 7.61 (4H, m, H6, H4, H2′, H6′), 7.53 (2H, m, H3′, H5′), 7.32–7.21 (10H, m, Ar-phenyl), 5.17 (1H, d, *J* = 11.4 Hz, NH), 4.92 (1H, d, *J* = 12.3 Hz, OCH_2), 4.86 (1H, d, *J* = 12.3 Hz, OCH_2), 4.47 (1H, q, *J* = 5.1 Hz, NCH), 3.23 (1H, dd, *J* = 13.9 Hz, *J* = 4.5 Hz, CH_2), 2.89 (1H, dd, *J* = 13.9 Hz, *J* = 10.5 Hz, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 163.3, 156.0, 148.2, 138.1, 137.3, 137.0, 135.4, 129.2, 129.0, 128.8, 128.6, 128.4, 128.2, 128.0, 127.9, 127.7, 126.8, 126,6, 123.4, 122.3, 65.7, 55.8, 36.1. Anal. Calcd for $C_{30}H_{24}Cl_2N_2O_5$ (563.43): C, 63.95; H, 4.29; N, 4.97. Found: C, 64.30; H, 4.65; N, 5.35.

(R)-5-Chloro-2-(4-chlorophenylcarbamoyl)phenyl 2-(benzyloxycarbonylamino)-3-phenylpropanoate (**3dd**). White solid; yield 44%; mp 181–184 °C; $[\alpha]_D^{26}$ 18.2 (*c* 2.7, $CHCl_3$). IR (KBr pellet): 3328, 1763 (CO ester), 1705, 1657, 1597, 1534, 1493, 1455, 1401, 1316, 1258, 1193, 1143, 1093, 1079, 1056, 908, 826, 743, 699, 508 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6) δ 8.31 (1H, br s, NH), 10.59 (1H, d, *J* = 7.8 Hz, H3), 7.73 (4H, m, H6, H4, H2′, H6′), 7.53 (2H, m, H3′, H5′), 7.39–7.22 (10H, m, Ar-phenyl), 4.95 (1H, d, *J* = 10.5 Hz, NH), 4.94 (2H, m, OCH_2), 4.50–4.43 (1H, m, NCH), 3.20 (1H, dd, *J* = 13.8 Hz, *J* = 4.5 Hz, CH_2), 2.89 (1H, dd, *J* = 13.8 Hz, *J* = 10.8 Hz, CH_2). ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.2, 163.3, 156.2, 148.4, 138.1, 137.4, 137.0, 135.5, 129.3, 129.0, 128.8, 128.7, 128.5, 128.2, 128.0, 127.8, 127.6, 126.8, 126.6, 123.3, 122.3, 65.7, 55.8, 36.1. Anal. Calcd for $C_{30}H_{24}Cl_2N_2O_5$ (563.43): C, 63.95; H, 4.29; N, 4.97. Found: C, 64.15; H, 4.40; N, 5.00.

10.2. HPLC determination of capacity factor *k*/calculated log *k*

The HPLC separation module Waters Alliance 2695 XE and Waters Photodiode Array Detector 2996 (Waters Corp., Milford, MA, USA) were used. The chromatographic column Symmetry® C_{18} 5 μm, 4.6 × 250 mm, Part No. WAT054275, (Waters Corp., Milford, MA, USA) was used. The HPLC separation process was monitored by Millennium32® Chromatography Manager Software, Waters 2004 (Waters Corp., Milford, MA, USA). The mixture of MeOH p.a. (70.0%) and H_2O-HPLC—Mili-Q Grade (30.0%) was used as a mobile phase. The total flow of the column was 1.0 mL/min, injection 30 μL, column temperature 45 °C and sample temperature 10 °C. The detection wavelength 210 nm was chosen. The KI methanolic solution was used for dead time (t_D) determination. Retention times (t_R) were measured in minutes.[20]

The capacity factors *k* were calculated using Millennium32® Chromatography Manager Software according to formula $k = (t_R - t_D)/t_D$, where t_R is the retention time of the solute, whereas t_D denotes the dead time obtained via an unretained analyte. Log *k*, calculated from the capacity factor *k*, is used as the lipophilicity index converted to log *P* scale. The log *k* values of individual compounds are shown in Table 1.

10.3. Lipophilicity calculations

Log *P*, that is, logarithm of the partition coefficient for *n*-octanol/water, was calculated using programs CS ChemOffice, CHEMDRAW Ultra ver. 10.0 (CambridgeSoft, Cambridge, MA, USA), and ACD/log *P* ver. 1.0 (Advanced Chemistry Development Inc., Toronto, Canada). Clog *P* values (the logarithm of *n*-octanol/water partition coefficient based on established chemical interactions) were generated by means of CS ChemOffice Ultra ver. 10.0 (CambridgeSoft, Cambridge, MA, USA) software. The results are shown in Table 1.

10.4. Antimycobacterial evaluation

The in vitro antimycobacterial activity of all prepared compounds was evaluated against *M. tuberculosis* CNCTC My 331/88 (dilution of the strain was 10^{-3} μmol/L), *M. kansasii* CNCTC My 235/80 (dilution of the strain was 10^{-4} μmol/L), *M. kansasii* 6509/96 (dilution of the strain was 10^{-4} μmol/L), and *M. avium* CNCTC My 330/88 (dilution of the strain was 10^{-5} μmol/L) in the National Reference Laboratory for *M. kansasii*, Regional Institute of Hygiene, Ostrava, Czech Republic. All strains were obtained from the Czech National Collection of Type Cultures (CNCTC), except *M. kansasii* 6509/96, which was clinically isolated. Antimycobacterial activities were determined in the Sula semisynthetic medium (SEVAC, Prague, Czech Republic). The tested compounds were added to the medium as dimethyl sulfoxide solutions. The following concentrations were used: 250, 125, 62, 32, 16, 8, 4, 2, and 1 μmol/L. The MIC values were determined after incubation at 37 °C for 7, 14, and 21 days. MIC (μmol/L) was the lowest substance concentration at which the inhibition of growth of mycobacteria occurred, see Table 1.

10.5. Study of hydrolytic behavior

Hydrolysis was performed on a Hewlett Packard UV/vis 8435 Diode Array apparatus in 10 mm closable quartz cell. The temperature during all experiments was 37 °C. The compounds were tested in methanolic solution (10 or 20 μL), which was added into the tempered solution of phosphate buffer (2 mL) with defined pH (7.4 or 5.5). Rate constants k_{obs} (s^{-1}) (pseudo-first-order) were calculated from the experimental data using the equation: $k_{obs}t$ = ln -ΔA + const., where $\Delta A = (A_\infty - A_t)$ or $(A_t - A_\infty)$.

10.6. Cytotoxic assay

The cytotoxic effect of the compounds was tested by XTT assay on human intestinal cell line HCT-8 (ECACC, UK). The cells were grown in EMEM supplemented by 5% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2. For the experiments, the cells were harvested with trypsin, resuspended in a fresh medium to a final concentration of 2×10^5 cells/mL, and seeded in aliquots (100 μL) onto 96-well tissue culture plates (TPP AG, Switzerland). The medium was removed after 24 h of cell incubation and replaced by EMEM culture containing the tested compounds dissolved in DMSO. In control wells, the cells were incubated in a medium containing DMSO without the tested compound (positive control for cell viability) and in the medium containing 20% DMSO (positive control for cytotoxic effect). The ability of the compounds to inhibit cellular growth was determined after 72 h by adding XTT solution (20 μL, 1 mg/mL, F. Hoffmann-La Roche Ltd, Switzerland) to each well. After incubation for 4 h absorbance was read at 450 nm using multiplate spectrometer LM 01 A (Beckmann Coulter Inc., USA). Each concentration of the compounds was tested in triplicates.

10.7. QSAR analysis

Quantitative structure analyses relationships between antituberculotic activity (log MIC) against *M.* tuberculosis (*M. tbc.*) 331/88, *M. avium* (330/88), *M. kansasii* (235/80, and clinically isolated 6509/96) after 21 days of incubation and Free-Wilson binary descriptors, experimentally determined log *k*, Hammett polar constants σ, and Taft inductive σ^* were investigated using the Intelligent Problem Solver (IPS) in Trajan 6.0 and this method is completely described in Supplementary data. The IPS automatically generates and examines different Artificial Neural Networks (ANNs) and observes their statistical performance.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.04.008.

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